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(57) Abstract

Lipases comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, which pocket forms part of and is surrounded by a lipid contact zone, are mutated by deletion or substitution of one or more amino acid residues in the lipid contact zone so as to change the electrostatic charge and/or hydrophobicity of the lipid contact zone or so as to change the surface conformation of the lipid contact zone of the lipases.

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LIPASE VARIANTS

FIELD OF INVENTION

The present invention relates to novel lipase enzyme variants with improved properties, DNA constructs coding for the expression of said variants, host cells capable of expressing the variants from the DNA constructs, as well as a method of producing the variants by cultivating said host cells.

BACKGROUND OF THE INVENTION

The advent and development of recombinant DNA techniques has had a profound influence on the field of protein chemistry. It has been envisaged that these techniques will make it possible to design peptides and proteins, such as enzymes, in accordance with specific criteria, thus permitting the production of compounds with desired properties.

Due to the availability of such techniques, it has become possible to construct enzymes with desired amino acid sequences, and a fair amount of research has been devoted to this object.

The primary structure of a number of lipases has been determined and described in the litterature (Boel et al., Lipids **23**, 701-706 (1988), de Caro et al., Biochim. Biophys. Acta **671**, 129-138 (1981), Winkler et al., Nature **343**, 771-774 (1990)). Furthermore also the tertiary structure of a more limited number of lipases has been elucidated (Winkler et al., Nature **343**, 771-774 (1990), Brady et al., Nature **343**, 767-770 (1990) J.D. Schrag et al., Nature 351, 1991, pp. 761-764). From these investigations it appears that lipases seem to have certain structural features in common, but that,on the other hand, major structural variations also xist among the lipases.

SUMMARY OF THE INVENTION

Further investigations have now shown that improved properties of lipases may be obtained by one or more specific mutations in the DNA sequence expressing a specific lipase in order to obtain lipase variants exhibiting such improved properties.

Consequently, in one aspect, the present invention relates to a lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, wherein the electrostatic charge and/or hydrophobicity of the lipid contact zone of the parent lipase is changed by deleting or substituting one or more negatively charged amino acid residues by neutral or positively charged amino acid residue(s), and/or by substituting one or more neutral amino acid residues by positively charged amino acid residue(s), and/or by deleting or substituting one or more hydrophilic amino acid residues by hydrophobic amino acid residue(s). For the sake of convenience, this lipase variant is termed lipase variant I in the following.

In the present context, the term "trypsin-like" is intended to indicate that the parent lipase comprises a catalytic triad at the active site corresponding to that of trypsin, i.e. the amino acids Ser, His and one of Asp, Glu, Asn or Gln. Some lipases may also comprise a surface loop structure which covers the active serine when the lipase is in inactive form (an example of such a lipase is described by Brady et al., Nature 343, 1990, pp. 767-770). When the lipase is activated, the loop structure is shifted to expose the active site residues, creating a surface with increased surface hydrophobicity which interacts with the lipid substrate at or during hydrolysis. For the present purpose, this surface is termed the "lipid contact zone", intended to include amino acid residues located within or forming part of this surface (or a corresponding surface of lipases which do not comprise such a loop structure). These residues may participate in lipase interaction with the

substrate at or during hydrolysis wh re the lipase hydrolyses triglycerides from the lipid phase when activated by contact with the lipid surface. During hydrolysis of the triglycerides, fatty acids and mono- and di-glycerides are formed in varying amounts. One reason for changing the electrostatic charge and/or hydrophobicity of the lipid contact zone by mutating the lipase in that zone is that the fatty acids formed during hydrolysis may remain in the lipid phase, thus forming a negatively charged surface. When the lipase is used for washing purposes, negatively charged detergents may form negative charges on the lipid surface. Thus, by preparing lipase variants which are less negatively charged and/or more hydrophobic, it may be possible to obtain lipases with different specificities and/or improved properties.

In another aspect, the present invention relates to a lipase variant comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, said lipase variant being further characterized by substitution, deletion, or insertion of one or more amino acid residues at the position of one or more of the amino acid residues constituting the sequence of the lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues participate in the interaction with the substrate at or during hydrolysis so as to change the surface conformation of said lipid contact zone. The purpose of such a surface modification of the lipase molecule is to provide improved accessibility of the active site of the lipase to a lipid substrate. For the sake of convenience, this lipase variant is termed lipase variant II in the following.

In a still further aspect, the invention relates to a lipase variant of a type comprising (i) a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule and (ii) a surface loop structure which covers the active serine

wh n the lipase is in inactive form and which changes its conformation when the lipase is activated so as to make the active serine accessible to a lipid substrate, the loop structure having a predominantly hydrophobic inner surface facing the binding pocket and a predominantly hydrophilic outer surface, said lipase variant being characterized by substitution, deletion or insertion of one or more amino acid residues at the position of one or more of the amino acid residues constituting the sequence of the loop structure and/or constituting the sequence of the lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues participate in either shifting the surface loop structure or the interaction with the substrate at or during hydrolysis. This will cause the loop structure to become more open whereby the active serine becomes more accessible to the substrate. For the sake of convenience, this lipase variant is termed lipase variant III in the following.

The present invention also relates to a DNA construct comprising a DNA sequence encoding a lipase variant as indicated above, a recombinant expression vector carrying said DNA construct, a cell transformed with the DNA construct or the expression vector, as well as a method of producing a lipase variant of the invention by culturing or growing said cell under conditions conducive to the production of the lipase variant, after which the lipase variant is recovered from the culture.

The invention further relates to a detergent additive comprising a lipase variant of the invention, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme, as well as to a detergent composition comprising lipase variant of the invention.

DETAILED DISCLOSURE OF THE INVENTION

In the present description and claims, the following abbreviations are used:

Amino acids:

A	=	Ala	=	Alanine
V	=	Val	=	Valine
L	=	Leu	=	Leucine
I	=	Ile	=	Isoleucine
P	=	Pro	=	Proline
F	=	Phe	=	Phenylalanine
W	=	Trp	=	Tryptophan
M	=	Met	=	Methionine
G	=	Gly	=	Glycine
S	=	Ser	=	Serine
T	=	Thr	=	Threonine
C	=	Cys	=	Cysteine
Y	=	Tyr	=	Tyrosine
N	=	Asn	=	Asparagine
Q	=	Gln	=	Glutamine
D	=	Asp	=	Aspartic Acid
E	=	Glu	=	Glutamic Acid
K	=	Lys	=	Lysine
R	=	Arg	=	Arginine
H	=	His	=	Histidine

In describing lipase variants according to the invention, the following nomenclature is used for ease of reference:

Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of glutamic acid for glycine in position 195 is shown as:

Gly 195 Glu or G195E

a deletion of glycine in the same position is shown as:

Gly 195 * or G195*

and insertion of an additional amino acid residue such as lysine is shown as:

Gly 195 GlyLys or G195GK

Where a specific lipase contains a "deletion" in comparison with other lipases and an insertion is made in such a position this is indicated as:

* 36 Asp or *36D

for insertion of an aspartic acid in position 36

Multiple mutations are separated by pluses, i.e.:

Arg 170 Tyr + Gly 195 Glu or R170Y+G195E

representing mutations in positions 170 and 195 substituting tyrosine and glutamic acid for arginine and glycine, respectively.

According to the invention, lipase variant I is preferably one in which one or more glutamic acid or aspartic acid residues of the lipid contact zone of the lipase are substituted by glutamine, asparagine, alanine, leucine, valine, serine, threonine, lysine, or arginine.

Although the parent lipase may be derived from a variety of sources such as mammalian lipases, e.g. pancreatic, gastric, hepatic or lipoprotein lipases, it is generally preferred that it is a microbial lipase. As such, the parent lipase may be selected from yeast, e.g. Candida, lipases, bacterial, e.g. Pseudomonas, lipases or fungal, e.g. Humicola or Rhizomucor lipases. It is particularly preferred to select the parent lipase from a group of structurally homologous lipases.

In a preferred embodiment of lipase variant I of the invention, the parent lipase is a Rhizomucor miehei lipase, in particular the lipase described in EP 305 216. In this embodiment, one or more negatively charged amino acid residues may be substituted by one or more positively charged or neutral amino acid residues as follows

D91N,K,R,A,V,L,S,T;
D256N,K,R,A,V,L,S,T;
D226N,K,R,A,V,L,S,T;
D61N,K,R,A,V,L,S,T;
D113N,K,R,A,V,L,S,T;
E201Q,K,R,A,V,L,S,T;
D243N,K,R,A,V,L,S,T.

In another preferred embodiment of lipase variant I of the invention, the parent lipase is a Humicola lanuginosa lipase, in particular the lipase produced by H. lanuginosa strain DSM 4106 (cf. EP 258 068). In this embodiment, one or more negatively charged amino acid residues may be substituted by one or more neutral or positively charged amino acid residues as follows:

E87Q,K,R,A,N,T,S,L,V;
D254N,K,R,A,Q,T,S,L,V;
D242N,K,R,A,Q,T,S,L,V;
E210Q,K,R,A,N,T,S,L,V;
E56Q,K,R,A,N,T,S,L,V;
D96N,K,R,A,Q,T,S,L,V;
D111N,K,R,A,Q,T,S,L,V;
D62A,Q,N,T,S,K,R,L,V;
E219A,Q,N,T,S,K,R,L,V;
E234A,Q,N,T,S,K,R,L,V;
E57A,Q,N,T,S,K,R,L,V
E99A,Q,N,T,S,K,R,L,V;
D27A,Q,N,T,S,K,R,L,V; or
E239A,Q,N,T,S,K,R,L,V.

Particularly preferred substitutions according to the invention are

E87Q + D254N + D242N + E210Q;
E87Q + D254N + E210Q;
D96N + E87Q + D254N:
R209A + E210A.

Alternatively, one or more neutral amino acid residues may be substituted by one or more positively charged amino acid residues as follows:

T267K,R;
S85K,R;
T226K,R;
N88K,R;
N92K,R;
I255K,R;
I202K,R
L206K,R;
L259K,R;
V203K,R; or
L227K,R

It should be noted that the Humicola lanuginosa lipase and the Rhizomucor miehei lipase belong to the same group of lipases. This implies that the overall three-dimensional structure of the two lipases is very similar and has been shown by X-ray crystallography to be highly homologous (a computer model of the H. lanuginosa and the Rh. miehei lipase is shown in Figs. 1A and B and 2A and B, respectively, from which the similarities between the lipid contact zones of the two lipases are clearly apparent). It is therefore probable that modifications of the type indicated for either lipase will also be functional for the other lipase.

In one embodiment of lipase variant II, one or more amino acid residues may be substituted by one or more other, less bulky amino acid residues. The purpose of such modification is to expose the active site of the lipase, thus making it more available for contact with the substrate. In particular, the less bulky amino acid residues may be selected from valine, threonine, serine, glycine or alanine.

Although the parent lipase of lipase variant II may be derived from a variety of sources such as mammalian lipases, e.g.

pancreatic, gastric, hepatic or lipoprotein lipases, it is generally preferred that it is a microbial lipase. As such, the parent lipase may be selected from yeast, e.g. Candida, lipases, bacterial, e.g. Pseudomonas, lipases or fungal, e.g. Humicola or Rhizomucor, lipases.

For instance, when the parent lipase is the Rhizomucor miehei lipase mentioned above, the following substitutions may preferably be made

I204V,A,T,S,G;
L208V,A,T,S,G;
F213V,A,T,S,G; or
I254V,A,T,S,G.

When the parent lipase is the Humicola lanuginosa lipase mentioned above, the following substitutions may preferably be made

I202V,A,T,S,G;
L206V,A,T,S,G;
F211V,A,T,S,G,I; or
I255V,A,T,S,G.

When lipase variant II is one provided with one or more surface loop sequences, one or more amino acid residues forming part of the loop sequence may advantagously be deleted. The purpose of such a modification is to improve the accessibility of the active serine to the substrate.

To this end, it has been found convenient to delete 2-8, in particular 2-6 amino acid residues from the loop sequence. For instance, when the parent lipase is the Rhizomucor miehei lipase mentioned above, one or more amino acid residues may be deleted at one or more of the following positions: 82-113, 211-215, 235-243, 245-269 or 264-269. Specific examples of suitable deletions (and, in the cas of the first xample mentioned, substitution) are as follows

N264* + T265* + G266* + L267* + C268* + T269* + C22T;

F213* + F215*;
D238* + L239* + E240* + D243*; or
S247* + F251* + T252*.

When the parent lipase is the Humicola lanuginosa lipase mentioned above, one or more amino acid residues may be deleted at one or more of the following positions: 84-112, 209-213, 238-245, 247-254 or 264-269. Specific examples of suitable deletions (and, in the case of the first example mentioned, substitution) are as follows

L264* + I265* + G266* + T267* + C268* + L269* + C22T;
R209* + E210*;
F211* + Y213*;
D242* + E239* + I241; or
N247* + D254*.

In a particular embodiment of lipase variant II, the parent lipase comprises a surface loop structure which covers the active serine when the lipase is in inactive form and which changes its conformation when the lipase is activated so as to make the active serine accessible to a lipid substrate. This loop structure has a predominantly hydrophobic inner surface facing the binding pocket and a predominantly hydrophilic outer surface. This lipase variant is characterized by deletion of one or more amino acid residues of the sequence composing the loop structure. The loop structure corresponds to the one described for human pancreatic lipase (cf. Winkler et al., Nature 343, 1990, pp. 771-774) and for Rhizomucor miehei lipase (cf. Brady et al., Nature 343, 1990, pp. 767-770). However, the lipase variant preferably has a shorter loop structure comprising no less than 5 amino acid residues. The loop structure may additionally comprise substitution of one or more amino acid residues. There is some indication, however, that the tryptophan residue present in the loop structure (W89 of the H. lanuginosa lipase and W88 of the Rh. miehei lipase) should be conserved.

In lipase variant III, at least one amino acid residue of the loop structure is preferably substituted by cysteine, and at least one other amino acid residue is preferably also substituted by cysteine, the two cysteine residues being so positioned relative to each other that they form a disulphide bond. This will cause the surface loop structure to shift so that it becomes more open whereby the active serine becomes more accessible to the substrate.

For instance, when the parent lipase is the Rhizomucor miehei lipase mentioned above, the following substitutions may be made

S114C + A90C;
R86C + D61C;
S84C + D61C;
N87C + D61C;
Y60C + R78C; or
Y60C + N87C.

When, on the other hand, the parent lipase is the Humicola lanuginosa lipase mentioned above, the following substitutions may be made

G61C + N88C;
G61C + E87C;
D62C + E87C;
D62C + S85C;
D62C + N88C; or
S116C + G91C.

Alternatively, a more open conformation of the loop structure in an aqueous medium may be obtained by substituting one or more hydrophilic amino acid residues by one or more less hydrophilic amino acid residues of the binding pocket in which the catalytic triad, including the active serine, is located.

For instance, when the parent lipase is the Rhizomucor miehei lipase mentioned above, the following substitutions may be made

I204V,A,T,S,G;

L208V,A,T,S,G;
F213V,A,T,S,G;
I254V,A,T,S,G;
L255V,A,T,S,G;
L258V,A,T,S,G;
L267V,A,T,S,G; or
F94L,T,K.

In particular, the amino acid substitutions may be combined as follows

I204T + L255T + L267T; or
L208T + I254T + L258T.

When the parent lipase is the Humicola lanuginosa lipase mentioned above, the following substitutions may be made

L93V,T,S,A,G;
I90V,T,S,A,G;
I86V,T,S,A,G;
I202V,T,S,A,G;
L206V,T,S,A,G;
I255V,T,S,A,G;
L259V,T,S,A,G;
F95L,T,K; or
F211L,T,K.

In preferred embodiments of lipase variant IV, one or more amino acid residues may be substituted as follows:

F95K;
I86T;
I90T;
I255T;
L259T;
L206T; or
L206T + I255T + L259T

It should be noted that, according to the invention, any one of the modifications of the amino acid sequence indicated above for

the lipase variants I-III may be combined with any one of the other modifications mentioned above.

Methods of preparing lipase variants of the invention

Several methods for introducing mutations into genes are known in the art. After a brief discussion of cloning lipase-encoding DNA sequences, methods for generating mutations at specific sites within the lipase-encoding sequence will be discussed.

Cloning a DNA sequence encoding a lipase

The DNA sequence encoding a parent lipase may be isolated from any cell or microorganism producing the lipase in question by various methods, well known in the art. First a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the lipase to be studied. Then, if the amino acid sequence of the lipase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify lipase-encoding clones from a genomic library of bacterial DNA, or from a fungal cDNA library. Alternatively, a labelled oligonucleotide probe containing sequences homologous to lipase from another strain of bacteria or fungus could be used as a probe to identify lipase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying lipase-producing clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming lipase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for lipase. Those bacteria containing lipase-bearing plasmid will produce colonies surrounded by a halo of clear agar, due to digestion of the substrate by secreted lipase.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., The EMBO J. 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA sequence, in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

Site-directed mutagenesis of the lipase-encoding sequence

Once a lipase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the lipase-encoding sequence, is created in a vector carrying the lipase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984, Biotechnology 2:646-639). U.S. Patent number 4,760,025, by Estell et al., issued July 26, 1988, discloses the introduction of oligonucleotides encoding multiple

mutations by performing minor alterations of the cassette, however, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into lipase-encoding sequences is described in Nelson and Long, Analytical Biochemistry 180, 1989, pp. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid (see also Figs. 3 and 4 where this method is further outlined).

Expression of lipase variants

According to the invention, a mutated lipase-coding sequence produced by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the lipase-coding sequence. For expression under the direction of control sequences, a target gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can support the transcription of the mutant lipase gene, include but are not limited to the prokaryotic ß-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can

also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

According to one embodiment B. subtilis is transformed by an expression vector carrying the mutated DNA. If expression is to take place in a secreting microorganism such as B. subtilis a signal sequence may follow the translation initiation signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the cell wall where it is cleaved from the product upon secretion. The term "control sequences" as defined above is intended to include a signal sequence, when is present.

In a currently preferred method of producing lipase variants of the invention, a filamentous fungus is used as the host organism. The filamentous fungus host organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of Aspergillus sp., such as A. niger, A. nidulans or A. oryzae. The use of A. oryzae in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

For expression of lipase variants in Aspergillus, the DNA sequence coding for the lipase variant is preceded by a promoter. The promoter may be any DNA sequence exhibiting a strong transcriptional activity in Aspergillus and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α-amylase, A. niger acid stable α-amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease or A. oryzae triose phosphate isomerase.

In particular when the host organism is A. oryzae, a preferred promoter for use in th process of the present invention is the A. oryzae TAKA amylase promoter as it exhibits a strong transcriptional activity in A. oryzae. The sequence of the TAKA amylase promoter appears from EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform a fungal host cell may suitably be as described in EP 238 023.

To ensure secretion of the lipase variant from the host cell, the DNA sequence encoding the lipase variant may be preceded by a signal sequence which may be a naturally occurring signal sequence or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. In particular, the signal sequence may be derived from a gene encoding an Aspergillus sp. amylase or glucoamylase, a gene encoding a Rhizomucor miehei lipase or protease, or a gene encoding a Humicola cellulase, xylanase or lipase. The signal sequence is preferably derived from the gene encoding A. oryzae TAKA amylase, A. niger neutral α-amylase, A. niger acid-stable α-amylase or A. niger glucoamylase.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing Aspergillus cells. The transformants are usually stable and may be cultured in the absence of selection pressure. However, if the transformants are found to be unstable, a selection marker introduced into the cells may be used for selection.

The mature lipase protein secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium

sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

The present invention also relates to a detergent additive comprising a lipase variant according to the invention, preferably in the form of a non-dusting granulate, stabilized liquid or protected enzyme. Non-dusting granulates may be produced e.g. according to US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238 216.

The detergent additive may suitably contain 0.02-200 mg of enzyme protein per gram of the additive. It will be understood that the detergent additive may further include one or more other enzymes, such as a protease, cellulase, peroxidase or amylase, conventionally included in detergent additives.

In a still further aspect, the invention relates to a detergent composition comprising a lipase variant of the invention. Detergent compositions of the invention additionally comprise surfactants which may be of the anionic, non-ionic, cationic, amphoteric, or zwitterionic type as well as mixtures of these surfactant classes. Typical examples of suitable surfactants are linear alkyl benzene sulfonates (LAS), alpha olefin sulfonates (AOS), alcohol ethoxy sulfates (AEOS), alcohol ethoxylates (AEO), alkyl sulphates (AS), alkyl polyglycosides (APG) and alkali metal salts of natural fatty acids.

Detergent compositions of the invention may contain other detergent ingredients known in the art as e.g. builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering

agents, anti soil-redeposition agents, perfumes, enzyme stabilizers, etc.

The detergent composition of the invention may be formulated in any convenient form, e.g. as a powder or liquid. The enzyme may be stabilized in a liquid detergent by inclusion of enzyme stabilizers as indicated above. Usually, the pH of a solution of the detergent composition of the invention will be 7-12 and in some instances 7.0-10.5. Other detergent enzymes such as proteases, cellulases, peroxidases or amylases may be included the detergent compositions of the invention, either separately or in a combined additive as described above.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is described in the following with reference to the appended drawings, in which

Fig. 1A and B are computer models showing the three-dimensional structure of the lipid contact zone of the H. lanuginosa lipase when the lipase is in inactive (A) and active (B) form, respectively. "White" residues represent hydrophobic amino acids (Ala, Val, Leu, Ile, Pro, Phe, Trp, Gly and Met), "yellow" residues represent hydrophilic amino acids (Thr, Ser, Gln, Asn, Tyr and Cys), "blue" residues represent positively charged amino acids (Lys, Arg and His), and "red" residues represent negatively charged amino acids (Glu and Asp);

Fig. 2A and 2B are computer models showing the three-dimensional structure of the lipid contact zone of the Rh. miehei lipase when the lipase is in inactive (A) and active (B) form, respectively. "White" residues represent hydrophobic amino acids (Ala, Val, Leu, Ile, Pro, Phe, Trp, Gly and Met), "yellow" residues represent hydrophilic amino acids (Thr, Ser, Gln, Asn, Tyr and Cys), "blue" residues represent positively charged amino acids (Lys, Arg and His), and "red" residues represent negatively charged amino acids (Glu and Asp);

Fig. 3 is a schematic representation of the preparation of plasmids encoding lipase variants by polymerase chain reaction (PCR);

Fig. 4 is a schematic representation of the three-step mutagenesis by PCR;

Fig. 5 shows a restriction map of plasmid pAO1;

Fig. 6 shows a restriction map of plasmid pAHL; and

Fig. 7 shows a restriction map of plasmid pARML.

The present invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

GENERAL METHODS

Expression of Humicola lanuginosa lipase and Rhizomucor miehei lipase in Aspergillus oryzae:

Cloning of Humicola lanuginosa lipase and Rhizomucor miehei lipase is described in EP 305,216 and EP 238 023, respectively. These patent applications also describe expression and characterization of the two lipases in Aspergillus oryzae. The two expression plasmids used are termed p960 (carrying the H. lanuginosa lipase gene) and p787 (carrying the R. miehei lipase gene).

The expression plasmids used in this application are identical to p787 and p960, except for minor modifications immediately 3' to the lipase coding regions. The modifications were made in the following way: p960 was digested with NruI and BamHI restriction enzymes. Between these two sites the BamHI/NheI fragment from plasmid pBR322, in which the NheI fragment was filled in with Klenow polymerase, was cloned, thereby creating plasmid pAO1

(Fig. 5) which contains unique BamHI and NheI sites. Between these unique sites BamHI/XbaI fragments from p960 and p787 were cloned to give pAHL (Fig. 6) and pARML (Fig. 7), respectively.

Site-directed in vitro mutagenesis of lipase genes:

Three different approaches were used for introducing mutations into the lipase genes.

One method employed was oligonucleotide site-directed mutagenesis which is described by Zoller & Smith, DNA, Vol. 3, No. 6, 479-488 (1984). The method is briefly described in the following, and is described thoroughly in example 1.

Isolated from the expression plasmid, the lipase gene of interest is inserted into a circular M13 bacteriophage vector. To the single-stranded genome, a chemically synthesized complementary DNA-strand is annealed. This DNA-strand contains the mutation to be introduced flanked by sequences complementary to lipase sequences on the circular DNA. In vitro, the primer is then extended in the entire length of the circular genome biochemically using Klenow polymerase. When transformed in E.coli, the heteroduplex will give rise to double-stranded DNA with the desired sequence from which a fragment can be isolated and re-inserted into the expression plasmid.

Another method employed is described in Nelson & Long, Analytical Biochemistry, 180, 147-151 (1989). It involves the 3-step generation of a PCR (polymerase chain reaction) fragment containing the desired mutation introduced by using a chemically synthesized DNA-strand as one of the primers in the PCR-reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation can be isolated by cleavage with restriction enzymes and re-inserted into the expression plasmid. This method is thoroughly described in example 3. In figures 3 and 4 the method is further outlined.

In a further method, usually termed "cassette mutagenesis", a segment between two restriction sites of the lipase-encoding region is replaced by a synthetic DNA fragment carrying the desired mutation.

Lipase Variants I

Example 1: Construction of a plasmid expressing the D96L variant of Humicola lanuginosa lipase.

Isolation of the lipase gene:

The expression plasmid p960 contains the coding region for Humicola lanuginosa lipase on a BamHI/XbaI restriction fragment (the DNA and amino acid sequence of the lipase are shown in the appended Sequence Listing ID No. 1). The BamHI/XbaI fragment was isolated as follows: The expression plasmid was incubated with the restriction endonucleases BamHI and XbaI. The conditions were: 5 μg plasmid, 10 units of BamHI, 10 units of XbaI, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT in 50 μl volume. The temperature was 37°C and the reaction time 2 hours. The two fragments were separated on a 1% agarose gel and the desired fragment was isolated from the gel.

Ligation to the vector M13mp18:

The bacteriophage vector M13mp18 on its double-stranded, replicative form was digested with BamHI and XbaI under conditions as described above. The isolated restriction fragment was ligated to the digested bacteriophage vector in the following reaction mixture: Fragment 0.2 μg, vector 0.02 μg, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP in a 20 μl volume at 16°C for 3 hours. 5 μl of this mixture was transformed into the E.coli strain JM101. The presence of fragment in the vector was identified by restriction enzyme analysis on double-stranded M13-DNA isolated from the transformants.

Isolation of single-stranded (ss) DNA (template):

From the transformant described above, ss-DNA was isolated according to a method described by Messing in Gene, 19, 269-276 (1982).

5' phosphorylation of the mutagenisation primer:

The mutagenisation primer with the sequence 5'-TTTCTTTCAACAAGAAGTTAAGA-3' was phosphorylated at the 5' end in a 30 µl reaction mixture containing 70 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 100 pmol oligonucleotide and 3.6 units of T4 polynucleotide kinase. The reaction was carried out for 30 min. at 37°C. Then, the enzyme was inactivated by incubating the mixture for 10 min. at 65°C.

Annealing of template and phosphorylated mutagenisation primer:

Annealing of template and primer was carried out in a 10 µl volume containing 0.5 pmol template, 5 pmol primer, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ 50 mM NaCl and 1 mM DTT by heating for 10 min. at 65°C and cooling afterwards to 0°C.

Extension/ligation reaction:

To the reaction mixture above, 10 µl of the following mixture was added: 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM TTP, 1 mM ATP, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 3 units of T4 DNA ligase and 2.5 units of Klenow polymerase. Then, the reaction was carried out for 16 hours at 16°C.

Transformation of JM101:

The reaction mixture above was transformed in different dilutions into $CaCl_2$-treated E.coli JM101 cells using standard techniques and plated in 2 x YT top agar on 2 x YT agar plates. (2 x YT = tryptone 16 g/l, yeast extract 10 g/l, NaCl 5 g/l. 2

x YT topagar = 2 x YT with 0.4% agarose added and autoclaved. 2 x YT agar plates = 2 x YT with 2% agar added and autoclaved). The plates were incubated at 37°C overnight.

Identification of positive clones:

The method used was plaque-lift hybridization which is described in the following: a nitrocellulose filter was placed on a plate with a suitable plaque-density, so that the filter was wetted. The filter was then bathed in the following solutions: 1.5 M NaCl, 0.5 M NaOH for 30 sec., 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0 for 1 min. and 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate) till later use. The filter was dried on 3MM filter paper and baked for 2 hours at 80°C in a vacuum oven.

The mutagenisation primer with the sequence 5'-TTTCTTTCAACAAGAAGTTAAGA-3' was labelled radioactively at the 5'-end in a 30 µl volume containing 70 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 10 pmol oligonucleotide, 20 pmol γ-32P-ATP and 3.5 units of T4 polynucleotide kinase. The mixture was incubated at 37°C for 30 min. and then for 5 min. at 100°C.

The dried filter was prehybridised for 2 hours at 65°C in 6 x SSC, 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidon, 0.2% sodium-dodecyl-sulphate (SDS) and 50 µg/ml sonicated salmon sperm DNA. Then, the reaction mixture containing the labelled probe was added to 15 ml of fresh pre-hybridization mix, and the filter was bathed herein overnight at 27°C with gentle shaking. After hybridisation, the filter was washed 3 times each 15 min. in 2 x SSC, 0.1% SDS and autoradiographed. After wash in the same solution, but now at 50°C, and another autoradiography, plaques containing DNA-sequences complementary to the mutagenisation primer were identified.

Because the identified clone is a result of a heteroduplex, the plaque was plated again. The hybridisation and identification steps were repeated.

Purification of double-stranded M13-phage DNA:

A re-screened clone was used for infection of E.coli strain JM101. A culture containing approximately 10^8 phages and 5 colonies of JM101 was grown for 5 hours in 5 ml 2 x YT medium at 37°C. Then, double-stranded, circular DNA was purified from the pellet according to a method described by Birnboim & Doly, Nucleic Acids Res., 2, 1513 (1979).

Isolation of a restriction fragment encoding modified lipase:

The DNA preparation (appr. 5 µg) isolated above was digested with 10 units of each of the restriction endonucleases BamHI and XbaI in 60 µl of 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 10 mM DTT for 2 hours at 37°C. The DNA products were separated on an agarose gel end the fragment was purified from the gel.

Ligation to the Aspergillus expression vector pAO1 (figure 5):

The isolated restriction fragment was ligated to the Aspergillus vector pAO1 digested with the restriction enzymes BamHI and NheI in the following reaction mixture: Fragment 0.2 µg, vector 0.02 µg, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP in a total volume of 20 µl. 5 µl of this reaction mix was used for transformation of E.coli strain MC1061, in which the modified expression plasmid was identified and propagated. The plasmid was called pAHLD96L and is identical to pAHL except for the modified codon.

Sequence verification of pAHLD96L:

The mutagenized plasmid was sequenced directly on the double-stranded plasmid using the dideoxy chain termination method originally described by Sanger.

Example 2: Construction of plasmids expressing other variants of Humicola lipase.

Other mutant lipase genes were constructed using the same method as described in example 1. Plasmid names and primers used for the modifications are listed below.

Plasmid name	Primer sequence
pAHLD96N	5'-TCTTTCAAGTTGAAGTTAAGA-3'
pAHLD111N	5'-GTGAAGCCGTTATGTCCCCTG-3'
pAHLE87Q	5'-CGATCCAGTTTTGTATGGAACGA-3'
pAHLR209A/E210A	5'-GCTGTAACCGAAAGCAGCCGGCGGGAGTCT-3'
pAHLE87A	5'-CGATCCAGTTAGCTATGGAACG-3'
pAHLE56A	5'-CTCCAGAGTCAGCAAACGAGTA-3'
pAHLE56Q	5'-CCAGAGTCTTGAAACGAGTAG-3'
pAHLD111L	5'-AAGTGAAGCCCAAATGTCCCCTG-3'
pAHLE210A	5'-TGTAACCGAAAGCGCGCGGCGG-3'
pAHLE210Q	5'-TAACCGAATTGGCGCGGCGGG-3'
pAHLR209A	5'-AACCGAATTCAGCCGGCGGGAGT-3'

Example 3: Construction of a plasmid expressing the D254N variant of Humicola lanuginosa lipase.

Linearization of plasmid pAHL:

The circular plasmid pAHL was linearized with the restriction enzyme SphI in the following 50 µl reaction mixture: 50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 µg plasmid and 2 units of SphI. The digestion was carried out for 2 hours at 37°C. The reaction mixture was extracted with phenol

(equilibrated with Tris-HCl, pH 7.5) and precipitated by adding 2 volumes of ice-cold 96% ethanol. After centrifugation and drying of the pellet, the linearized DNA was dissolved in 50 µl H_2O and the concentration estimated on an agarose gel.

3-step PCR mutagenesis:

As shown in Fig. 4, 3-step mutagenisation involves the use of four primers:

Mutagenisation primer (=A):
5'-GTGCGCAGGGATGTTCGGAATGTTAGG-3'

PCR Helper 1 (=B):
5'-GGTCATCCAGTCACTGAGACCCTCTACCTATTAAATCGGC-3'

PCR Helper 2 (=C): 5'-CCATGGCTTTCACGGTGTCT-3'
PCR Handle (=D): 5'-GGTCATCCAGTCACTGAGAC-3'

All 3 steps were carried out in the following buffer containing: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 2.5 units Taq polymerase.

In step 1, 100 pmol primer A, 100 pmol primer B and 1 fmol linearized plasmid was added to a total of 100 µl reaction mixture and 15 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C were carried out.

The concentration of the PCR product was estimated on an agarose gel. Then, step 2 was carried out. 0.6 pmol step 1 product and 1 fmol linearized plasmid was contained in a total of 100 µl of the previously mentioned buffer and 1 cycle consisting of 5 minutes at 95°C, 2 minutes at 37°C and 10 minutes at 72°C was carried out.

To th step 2 reaction mixture, 100 pmol primer C and 100 pmol primer D was added (1 µl of each) and 20 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C were carried out. This manipulation comprised step 3 in the mutagenisation procedure.

Isolation of mutated restriction fragment:

The product from step 3 was isolated from an agarose gel and re-dissolved in 20 µl H_2O. Then, it was digested with the restriction enzyme BspMII in a total volume of 50 µl with the following composition: 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT and 10 units of BspMII. Incubation was at 37°C for 2 hours. The 264 bp BspMIII fragment was isolated from an agarose gel.

Ligation to expression vector pAHL:

The expression plasmid pAHL was cleaved with BspMII under conditions indicated above and the large fragment was isolated from an agarose gel. To this vector, the mutated fragment isolated above was ligated and the ligation mix was used to transform E.coli. The presence and orientation of the fragment was verified by cleavage of a plasmid preparation from a transformant with restriction enzymes. Sequence analysis was carried out on the double-stranded plasmid using the di-deoxy chain termination procedure developed by Sanger. The plasmid was named pAHLD254N and is identical to pAHL, except for the altered codon.

Example 4: Construction of plasmids expressing other variants of Humicola lipase.

The following mutants were constructed using the same method as described in example 3, except other restriction enzymes were used for digesting the PCR-product and the vector used for

recloning of the mutated fragment. Plasmid names and primers used for the modifications are listed below.

Plasmid name	Primer A sequence
pAHLD254K	5'-GTGCGCAGGGATCTTCGGAATGTT-3'
pAHLD254R	5'-GTGCGCAGGGATTCTCGGAATGTT-3'
pAHLD242N	5'-GCCGCCGGTGGCGTTGATGCCTTCTAT-3'
pAHLD242N/D254N	5'GTGCGCAGGGATGTTCGGAATGTTAGGCTGGTTATTGC CGCCGGTGGCGTTGATGCCTTCTAT-3'
pAHLE87R	5'-CCCGATCCAGTTTCTTATCGATCGAGAGCCGCGG-3'
pAHLE87K	5'-CGATCCAGTTCTTTATCGATCGAGAGCCACGG-3'

Example 5: Construction of lipase variants by combination of available mutants:

The following mutants were constructed by combining plasmid fragments of mutants constructed above. For example, pAHLE87K/D254K was constructed by isolating the BamHI/BstXI restriction fragment from pAHLE87K and inserting the fragment into pAHLD254K digested with BamHI and BstXI:

Plasmid
pAHLE87K/D254K
pAHLE87Q/D254N/D242N/E210Q
pAHLE87Q/D242N/E210Q
pAHLR209A/E210A/D96L
pAHLR209A/E210Q/E56Q
pAHLE210Q/D242N/D254N
pAHLE87Q/E210Q/D242N

Lipase Variants II

Example 6: Construction of a plasmid expressing the ΔL264->L269 variant of Humicola lanuginosa lipase.

Linearization of plasmid pAHL:

The circular plasmid pAHL was linearized with the restriction enzyme SphI in the following 50 µl reaction mixture: 50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 µg plasmid and 2 units of SphI. The digestion was carried out for 2 hours at 37°C. The reaction mixture was extracted with phenol (equilibrated with Tris-HCl, pH 7.5) and precipitated by adding 2 volumes of ice-cold 96% ethanol. After centrifugation and drying of the pellet, the linearized DNA was dissolved in 50 µl H_2O and the concentration estimated on an agarose gel.

3-step PCR mutagenesis:

As shown in figure 4, 3-step mutagenisation involves the use of four primers:

Mutagenisation primer (=A):
5'-CAGGCGCGCCGGCCACCCGAAGTACCATAG-3'

PCR Helper 1 (=B):
5'-GGTCATCCAGTCACTGAGACCCTCTACCTATTAAATCGGC-3'

PCR Helper 2 (=C): 5'-CCATGGCTTTCACGGTGTCT-3'
PCR Handle (=D): 5'-GGTCATCCAGTCACTGAGAC-3

Helper 1 and helper 2 are complementary to sequences outside the coding region. All 3 steps were carried out in the following buffer containing: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 2.5 units Taq polymerase.

In step 1, 100 pmol primer A, 100 pmol primer B and 1 fmol linearized plasmid was added to a total of 100 µl reaction mixture and 15 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C were carried out.

The concentration of the PCR product was estimated on an agarose gel. Then, step 2 is carried out. 0.6 pmol step 1 product and 1

fmol linearized plasmid was contained in a total of 100 µl of the previously mentioned buffer and 1 cycle consisting of 5 minutes at 95°C, 2 minutes at 37°C and 10 minutes at 72°C was carried out.

To the step 2 reaction mixture, 100 pmol primer C and 100 pmol primer D is added (1 µl of each) and 20 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C were carried out. This manipulation comprised step 3 in the mutagenisation procedure.

Isolation of mutated restriction fragment:
The product from step 3 was isolated from an agarose gel and re-dissolved in 20 µl H_2O. Then, it was digested with the restriction enzymes BglII and BstXI in a total volume of 50 µl with the following composition: 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 10 units of BglII and 10 units of BstXI. Incubation was at 37°C for 2 hours. The 200 bp BglII/BstXI fragment was isolated from an agarose gel.

Ligation to expression vector pAHL:
The expression plasmid pAHL was cleaved with BglII and BstXI under conditions indicated above and the large fragment was isolated from an agarose gel. To this vector, the mutated fragment isolated above was ligated and the ligation mix was used to transform E.coli. The presence of the fragment was verified by cleavage of a plasmid preparation from a transformant with restriction enzymes. Sequence analysis was carried out on the double-stranded plasmid using the di-deoxy chain termination procedure developed by Sanger. The plasmid was named pAHLΔL264->L269 and is identical to pAHL, except for the deleted codons.

Example 7: Construction of plasmids expressing other variants of Humic la lipas .

The following mutants were constructed using the same method as described in example 6, with the exception that other restriction enzymes were used for digesting the PCR-product and the vector used for recloning of the mutated fragment. Plasmid names and primers used for the modifications are listed below.

Plasmid name	Primer A sequence
pAHLΔN247->D254	5'-TAGGTGCGCAGGGATCGGAATGTTAG GCTGGTTGCCGCCGGTGGCATC-3'
pAHLE239* + I241* + D242*	5'-ATTGCCGCCGGTGGCGCCTATCTTCA CGATATC-3'

Example 8: Construction of the lipase variant L206V by cassette mutagenesis:

Using the method outlined in example 6, the coding sequence on plasmid pAHLwas modified to contain unique AvrII and MluI sites. The AvrII site was made by changing the G681 of the coding sequence to an adenosine. The MluI site was made by changing C759 to G and A762 to T. The new plasmid was named pAHL7 and encodes the same lipase as pAHL. Between the AvrII- and MluI-sites the following synthetically made linker was inserted (changes the Leu-codon to a Val-codon and deletes the ScaI-site for easy screening among transformants):

```
                ***
 AvrII  CTAGGGTTCCGCCGCGCGAATTCGGTTACAGCCATTCT
          CCAAGGCGGCGCGCTTAAGCCAATGTCGGTAAGA
          ArgValProProArgGluPheGlyTyrSerHisSer -
          205          210              216
             *
    AGCCCAGAATACTGGATCAAATCTGGAACCCTTGTCCCCGTCA     MluI
    TCGGGTCTTATGACCTAGTTTAGACCTTGGGAACAGGGGCAGTGCGC
    SerProGluTyrTrpIleLysSerGlyThrLeuValProValThrArg
    217     220          225          230
```

The resulting plasmid was named pAHLL206V, and is identical to pAHL, except for the changed bases.

Example 9: Construction of other lipase variants using cassette mutagenesis:

Other mutants constructed by cassette mutagenesis as described in example 8 are listed below. Other linkers were used for introducing the appropriate mutations.

Plasmid name
pAHLL206A
pAHLF211V
pAHLF211A
pAHLDR209/E210

Lipase Variants III

Example 10: Construction of a plasmid expressing the D62C + E87C variant of Humicola lanuginosa lipase.

Linearization of plasmid pAHL:

The circular plasmid pAHL was linearized with the restriction enzyme SphI in the following 50 µl reaction mixture: 50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 µg plasmid and 2 units of SphI. The digestion was carried out for 2 hours at 37°C. The reaction mixture was extracted with phenol (equilibrated with Tris-HCl, pH 7.5) and precipitated by adding 2 volumes of ice-cold 96% ethanol. After centrifugation and drying of the pellet, the linearized DNA was dissolved in 50 µl H_2O and the concentration estimated on an agarose gel.

3-step PCR mutagenesis:

As shown in figure 4, 3-step mutagenisation involves the use of four primers:

Mutagenisation primer
(=A): 5'-ATTCCCGATCCAGTTACATATGGAACGAGAGCCACGGAAGCTTAGGACG
ATCAATTTGTTCGTGTTGTCGAGAGCAAGGAAGCCGGTGACACAGCCCACTC
CAGAGTC-3'

PCR Helper 1 (=B): 5'-GGTCATCCAGTCACTGAGACCCTCTACCTATTAAA-
TCGGC-3'

PCR Helper 2 (=C): 5'-CCATGGCTTTCACGGTGTCT-3'
PCR Handle (=D): 5'-GGTCATCCAGTCACTGAGAC-3'

Apart from altering two codons in the lipase coding region, primer A also introduces a silent mutation, thereby creating a HindIII-site between the codon-changes.

All 3 steps were carried out in the following buffer containing: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 2.5 units Taq polymerase.

In step 1, 100 pmol primer A, 100 pmol primer B and 1 fmol linearized plasmid was added to a total of 100 µl reaction mixture and 15 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C were carried out.

The concentration of the PCR product was estimated on an agarose gel. Then, step 2 is carried out. 0.6 pmol step 1 product and 1 fmol linearized plasmid was contained in a total of 100 µl of the previously mentioned buffer and 1 cycle consisting of 5 minutes at 95°C, 2 minutes at 37°C and 10 minutes at 72°C was carried out.

To the step 2 reaction mixture, 100 pmol primer C and 100 pmol primer D is added (1 µl of each) and 20 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C were carried out. This manipulation comprised step 3 in the mutagenisation procedure.

Isolation of mutated restriction fragment:
The product from step 3 was isolated from an agarose gel and re-dissolved in 20 µl H_2O. Then, it was digested with the restriction enzymes BamHI and BstXI in a total volume of 50 µl with the following composition: 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 10 units of BamHI and 10 units of BstXI. Incubation was at 37°C for 2 hours. The 733 bp BamHI/BstXI fragment was isolated from an agarose gel.

Ligation to expression vector pAHL:
The expression plasmid pAHL was cleaved with BamHI and BstXI under conditions indicated above and the large fragment was isolated from an agarose gel. To this vector, the mutated fragment isolated above was ligated and the ligation mix was used to transform E.coli. The presence and orientation of the fragment was verified by cleavage of a plasmid preparation from a transformant with restriction enzymes. Sequence analysis was carried out on the double-stranded plasmid using the dideoxy chain termination procedure developed by Sanger. The plasmid was named pAHLD62C/E87C and is identical to pAHL, except for the altered codons.

Example 11: Construction of plasmids expressing other variants of Humicola lipase.

The following mutants were constructed using the same method as described in example 10, except that other restriction enzymes were used for digesting the PCR-product and the vector used for recloning of the mutated fragment. Plasmid names and primers used for the modifications are listed below.

Plasmid name	Primer A sequence
pAHLG61C/E87C	5'-AAGATTCCCGATCCAACACTCTATGGAACGAGAGCCACGGAAG-CTTAGGACGATCAATTTGTTCGTGTTGTCGAGAGCAAGGAAGCCGG-TGACATCACACACTCCAGAGTCTTC-3'
pAHLI255T/L259T	5'-TAACCCGAAGTACCAAGTGTGCGCAGGAGTATCCGGA ATGTTAG-3'

Example 12: Construction of the lipase variant L206V by cassette mutagenesis:

Using the method outlined in example 3, the coding sequence on plasmid pAHL was modified to contain unique AvrII and MluI sites. The AvrII site was made by changing the G681 of the coding sequence to an adenosine. The MluI site was made by changing C759 to G and A762 to T. The new plasmid was named pAHL7 and encodes the same lipase as pAHL. Between the AvrII- and MluI-sites the following synthetically made linker was inserted (changes the Leu-codon to a Val-codon and deletes the ScaI-site for easy screening among clones with the linker cloned):

```
            ***
      CTAGGGTTCCGCCGCGCGAATTCGGTTACAGCCATTCT
         CCAAGGCGGCGCGCTTAAGCCAATGTCGGTAAGA
       ArgValProProArgGluPheGlyTyrSerHisSer -
       205           210              216
            *
  AGCCCAGAATACTGGATCAAATCTGGAACCCTTGTCCCCGTCA
  TCGGGTCTTATGACCTAGTTTAGACCTTGGGAACAGGGGCAGTGCGC
  SerProGluTyrTrpIleLysSerGlyThrLeuValProValThrArg
  217    220          225          230
```

The resulting plasmid was named pAHLL206V, and is identical to pAHL, except for the changed bases.

Example 13: Construction of other lipase variants using cassette mutagenesis:

Other mutants constructed by cassette mutagenesis as described in example 3 are listed below. Other linkers were used for introducing the appropriate mutations.

Plasmid name

pAHLL206T
pAHLL206S
pAHLL206A
pAHLL206G
pAHLF211L
pAHLF211T
pAHLF211K

Example 14: Construction of lipase variants by combination of available mutants:

The following mutants were constructed by combining plasmid fragments of mutants constructed above. For example, pAHLG61C+E87C was constructed by isolating the HindIII restriction fragment from pAHLD62C+E87C (the primer used for the construction introduced a HindIII site between the two mutations) and inserting the fragment into pAHLG61C+N88C digested with HindIII (also introduced together with the mutations):

Plasmid
pAHLD61C+E87C
pAHLL206S+I255T+L259T

Example 15

Transformation of Aspergillus oryzae (general procedure)

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) was inoculated with spores of A. oryzae and incubated with shaking for about 24 hours. The mycelium was harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium was suspended in 15 ml of 1.2 M $MgSO_4$, 10 mM NaH_2PO_4, pH = 5.8. The suspension was cooled on ice and 1 ml of buffer containing 120 mg of Novozym® 234, batch 1687 was added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) was added and incubation with gentle agitation continued for 1.5 - 2.5 hours at 37°C until a large number of

protoplasts was visible in a sample inspected under the microscope.

The suspension was filtered through miracloth, the filtrate transferred to a sterile tube and overlayed with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation was performed for 15 min. at 1000 g and the protoplasts were collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$) were added to the protoplast suspension and the mixture was centrifugated for 5 min. at 1000 g. The protoplast pellet was resuspended in 3 ml of STC and repelleted. This was repeated. Finally, the protoplasts were resuspended in 0.2 - 1 ml of STC.

100 µl of protoplast suspension was mixed with 5 - 25 µg of p3SR2 (an A. nidulans amdS gene carrying plasmid described in Hynes et al., Mol. and Cel. Biol., Vol. 3, No. 8, 1430-1439, Aug. 1983) in 10 µl of STC. The mixture was left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH = 7.5 was added and carefully mixed (twice) and finally 0.85 ml of the same solution was added and carefully mixed. The mixture was left at room temperature for 25 min., spun at 2.500 g for 15 min. and the pellet was resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts were spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4 - 7 days at 37°C spores were picked, suspended in sterile water and spread for single colonies. This procedure was repeated and spores of a single colony after the second reisolation were stored as a defined transformant.

Example 16

Expression of the lipase variant D96L in A. oryzae

pAHLD96L was transformed into A. oryzae IFO 4177 by cotransformation with p3SR2 containing the amdS gene from A. nidulans as described in example 15. Protoplasts prepared as described were incubated with a mixture of equal amounts of pAHLD96L and p3SR2, approximately 5 µg of each were used. 9 transformants which could use acetamide as sole nitrogen source were reisolated twice. After growth on YPD for three days, culture supernatants were analyzed using the assay for lipase activity described in example 16 (Purification of lipase variants of the invention). The best transformant was selected for further studies and grown in a 1 l shake-flask on 200 ml FG4 medium (3% soy meal, 3% maltodextrin, 1% peptone, pH adjusted to 7.0 with 4 M NaOH) for 4 days at 30°C. Under these conditions the transformant gave about 500 lipase units per ml of culture.

The other lipase variants were produced essentially as described above, using the general procedure described in example 15.

Example 17

Purification of lipase variants of the invention

Assay for lipase activity :
A substrate for lipase was prepared by emulsifying glycerine tributyrat (MERCK) using gum-arabic as emulsifier.
Lipase activity was assayed at pH 7 using pH stat method. One unit of lipase activity (LU/mg) was defined as the amount needed to liberate one micromole fatty acid per minute.

Step 1:- Centrifuge the fermentation supernatant, discard the precipitate. Adjust the pH of the supernatant to 7 and add gradually an equal volume of cold 96 % ethanol. Allow the mixture to stand for 30 minutes in an ice bath. Centrifuge and discard the precipitate.

Step 2:- Ion exchange chromatography. Filter the supernatant and apply on DEAE-fast flow (Pharmacia TM) column equilibrated with

50 mM tris-acetate buffer pH 7. Wash the c lumn with the same buffer till absorption at 280 nm is lower than 0.05 OD. Elute the bound enzymatic activity with linear salt gradient in the same buffer (0 to 0.5 M NaCl) using five column volumes. Pool the fractions containing enzymatic activity .

Step 3:- Hydrophobic chromatography. Adjust the molarity of the pool containing enzymatic activity to 0.8 M by adding solid Ammonium acetate. Apply the enzyme on TSK gel Butyl- Toyopearl 650 C column (available from Tosoh Corporation Japan) which was pre-equilibrated with 0.8 M ammonium acetate. Wash the unbound material with 0.8 M ammonium acetate and elute the bound material with distilled water.

Step 4:- Pool containing lipase activity is diluted with water to adjust conductance to 2 mS and pH to 7. Apply the pool on High performance Q Sepharose (Pharmacia) column pre-equilibrated with 50 mM tris -acetate buffer pH 7. Elute the bound enzyme with linear salt gradient.

Example 18

The washing performance of lipase variants of the invention

The washing performance of Humicola lanuginosa lipase variants of the invention was evaluated on the basis of the enzyme dosage in mg of protein per litre according to OD_{280} compared to the wild-type H. lanuginosa lipase.

Wash trials were carried out in 150 ml beakers placed in a thermostated water bath. The beakers were stirred with triangular magnetic rods.

The experimental conditions were as follows:

Method: 3 cycles with overnight drying between each cycle

Wash liquor:	100 ml per beaker	
Swatches:	6 swatches (3.5 x 3.5 cm) per beaker	
Fabric:	100% cotton, Test Fabrics style #400	
Stain:	Lard coloured with Sudan red (0.75 mg dye/g of lard). 6 µl of lard heated to 70°C was applied to the centre of each swatch. After application of the stain, the swatches were heated in an oven at 75°C for 30 minutes. The swatches were then stored overnight at room temperature prior to the first wash.	
Detergent:	LAS (Nansa 1169/P, 30% a.m.)	1.17 g/l
	AEO (Dobanol 25-7)	0.15 g/l
	Sodium triphosphate	1.25 g/l
	Sodium sulphate	1.00 g/l
	Sodium carbonate	0.45 g/l
	Sodium silicate	0.15 g/l
pH:	10.2	
Lipase conc.:	0.075, 0.188, 0.375, 0.75 and 2.5 mg of lipase protein per litre	
Time:	20 minutes	
Temperature:	30°C	
Rinse:	15 minutes in running tap water	
Drying:	overnight at room temperature (~20°C, 30-50% RH)	
Evaluation:	after the 3rd wash, the reflectance at 460 nm was measured.	

Results

Dose-response curves were compared for the lipase variants and the native H. lanuginosa lipase. The dose-response curves were calculated by fitting the measured data to the following equation:

$$\Delta R = \Delta R_{max} \frac{C^{0.5}}{K + C^{0.5}} \qquad (I)$$

where ΔR is the effect expressed in reflectance units
C is the enzyme concentration (mg/l)

ΔR_{max} is a constant expressing the maximum effect
K is a constant; K^2 expresses the enzyme concentration at which half of the maximum effect is obtained.

Based on the characteristic constants ΔR_{max} and K found for each lipase variant as well as the wild-type lipase, improvement factors were calculated. The improvement factor, defined as

$$f_{improve} = C_{WT}/C \qquad (II)$$

expresses the amount of lipase variant protein needed to obtain the same effect as that obtained with 0.25 mg/l of the reference wild-type protein (C_{WT}).

Thus, the procedure for calculating the improvement factor was as follows:

1) The effect of the wild-type protein at 0.25 mg/l ($\Delta R_{wild\text{-}type}$) was calculated by means of equation (I);
2) the concentration of lipase variant resulting in the same effect as the wild-type at 0.25 mg/l was calculated by means of the following equation:

$$C = \left(K_{(variant)} \frac{\Delta R_{(wild\text{-}type)}}{\Delta R_{max(variant)} - \Delta R_{(wild\text{-}type)}}\right)^2 \qquad (III)$$

3) the improvement factor was calculated by means of equation (II).

The results are shown in Table 1 below.

Table 1

Variant	Improvement factor
D96L	4.4
D111L	1.0
E87A	1.0
E56A	1.6
E56Q	2.6
R209A	1.1
D242N	1.7
R209A+E210A	1.9
R209A+E210A+D96L	2.8
E210Q+D242N+D254N	1.8
R209A+E210A+D96L+E56Q	1.5
L206A	1.0
L206V	1.6
L206S	1.3
F211L	1.0
F211I	1.1
F211A	
R209* + E210*	0.9

It appears from Table 1 that the lipase variants R209A+E210A, E56Q and D96L have a considerably better wash performance than the wild-type lipase. This might possibly be ascribed to the decreased negative charge and increased hydrophobicity of these variants resulting in increased adsorption during washing and consequently higher activity during the drying phase. The performance of the lipase variants E87A, D111N and R209A in on a par with that of the wild-type enzyme.

Example 19

Increased Thermostability of Lipase Variants

The thermostability of selected variants of H. lanuginosa lipase has been examined by Differential Scanning Calorimetry (DSC). Using this technique, the thermal denaturation temperature, Td,

is determined by heating an enzyme solution at a constant programmed rate.

Experiments:

The Differential Scanning Calorimeter, MC-2D, from MicroCal Inc. was used for the investigations. 50 mM buffer solutions in was prepared at the following pH-values: 4 (acetate), 7 (TRIS-acetate), 10 (glycine). The enzyme concentration ranged between 0.6 - and 0.9 mg/ml, and a total volume of ca. 1.2 ml was used for each experiment. All samples were heated from 5 'C to 95 'C at a scan rate of 90°C/hr..

Results:

The results for the wild type and selected mutants are shown in the table below.

		pH 4		pH 7		pH 10	
No	Mutation	Td	dTd	Td	dT_d	Td	dTd
WT	-	58.9	-	74.7	-	69.3	-
1	F211A	60.2	+1.3	75.8	+1.1	70.3	+1.0
2	T267R	59.4	+0.5	75.7	+1.0	70.0	+0.7
3	D111N	58.3	-0.6	75.6	+0.9	69.9	+0.6
4	F211L	57.8	-1.1	74.8	0.1	69.4	0.1

Note: dTd denotes the the change in thermostability as a result of the mutation.

Example 20

Storage stability of H. lanuginosa lipase variants in liquid detergent.

Several variants were tested in a model liquid detergent with the following composition:

			% w/w
	Anionic	LAS	10
		AS	1
		Soap	14
	Nonionic	AEO	13
	Solvent	1,2-propane diol	3
		Ethanol	5
	Buffer	TEA	6
	Builder	Sodium citrate	1
	Neutr.agent	NaOH	2
	Stabilizer etc.	SXS	1
		Ca^{2+}	0,0025
		Phosphonate	0,4
		Na_2SO_4	0,2
	Water	add to 100%	
	pH	8 or 10	

1000 LU per gram of detergent was added and in some samples 0.025 AU/g (Alcalase®) was added. Samples were stored according to the following scheme (triplicate of each):

Storage temperature:	-18°C	30°C
Detergent		
pH 8 , no protease	2 & 7 days	2 & 7 days
pH 8 , 0.025 AU/g		2 days
pH 10, no protease	7 days	7 days

Following this incubation the samples were analyzed according to the LU-method (Novo Nordisk AF 95.5).

Assuming that the decay of lipase activity follows a first order kinetic, the rate constant of the decay can be determined:

$A(t) = A_0*\exp(-k*t)$

A(t) being the enzyme activity at time t, A_0 the initial activity and k the first order rate constant.

For the detergent containing protease a rate constant for the proteolysis can be calculated from

$A(t) = A_0*\exp(-[k+k_p]*t)$

where k_p is the rate constant of proteolysis, and where k is calculated from the stability data determined in the detergent without protease.

In each experiment the wild-type H. lanuginosa lipase was included as a reference, and comparison of the variants with the wild-type is only done within an experiment in order to reduce the uncertainty of variation between experiments.

Below the results are given, and the relative improvement of a variant over the wild-type is given as:

$IF_x = k_{wt}/k_x$

where IF means Improvement factor, k_{wt} is the rate constant of decay of the wild-type (at the given conditions) and k_x is the corresponding rate constant of the variant in question in the same experiment.

IF expresses the relative improvement in half-life (IF_x=2 indicates that the half-life of variant x is twice as long as that of the wild-type in the same experiment).

Based on an estimation of variations of replicates within an experiment an IF < 0.7 or IF > 1.3 is considered significant.

The unit of k is $(day)^{-1}$.

Variant	Experiment no.	pH 8 no prot. k*)	IF*)	pH 8 +Alcalase k_p	IF	pH 10 no prot. k	IF
Wildtype	3	0.02		0.48		0.19	
	5	0.02		0.40		0.16	
	6	0.00		0.34		0.09	
	7	0.01		0.52		0.22	
	8 a	0.01		0.50		0.09	
	b	0.01		0.52		0.07	
D96N	3	0.00		0.21	2.3	0.15	1.3
	5	0.02		0.26	1.6	n.d.	
D111N	3	0.00		0.50	1.0	0.16	1.2
	5	0.02		0.31	1.3	0.13	1.2
E56Q	3	0.01		0.22	2.2	0.14	1.4
D96L	6	0.01		0.17	2.0	0.08	1.2
	7	0.00		0.23	2.3	0.09	2.6
R209A/E210A/D96L	7	0.02		0.36	1.4	0.10	2.3
E210Q/D242N/D254N	7	0.02		0.49	1.0	n.d.	
F211L	6	0.02		0.41	0.8	0.08	1.1
F211T	8	0.02		1.4	0.4	0.06	1.5
F211A	8	0.01		0.58	0.9	0.02	3.1
F211I	8	0.02		1.4	0.4	0.08	1.2

*)k in the detergent at pH 8 is in all cases very low, and due to the short storage time (7 days, approx. 90% residual activity) it is not determined very accurately. Hence the IF is not calculated.

In conclusion a number of the tested variants had improved resistance to proteolytic degradation, and they almost all had improved resistance to alkaline conditions.

Example 21

Specific activity

A higher specific activity (amounts of substrate molecules cleaved pr. unit time pr. unit amount) than the wild-type (wt) was measured for the lipase variants shown below. This means that these lipases have a superior performance of hydrolysing the actual substrate.

The lipases were fermented and purified in the same way. The purified lipases were tested in a standard LU assay (Analytical method, internal NOVO NORDISK number AF 95/6-GB 1991.02.07). The sample was analysed twice, and the mean values are tabulated. The amount of protein was estimated by optical density measurements on a Shimadzu spectrofotometer, using the wave-length 280 nm. The sample was regarded as pure when the proportional value of OD280 divided by OD260 was greater than 1.6, together with a single band SDS-polyacrylamid gel electroforesis.

Humicola lanuginosa	Specific activity LU/OD280
D111N	4290*
E56A	4890*
L206V	4750
F211T	4550
F211V	5060
F211I	6686
R209*/E210*	6686
R209A/E210A/D96L	4818
wt	3790

* only tested once

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Novo Nordisk A/S

(ii) TITLE OF INVENTION: Lipase Variants

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: DK 2196/90
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(A) APPLICATION NUMBER: DK 2194/90
(B) FILING DATE: 13-SEP-1990

(vii) PRIOR APPLICATION DATA:
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(B) FILING DATE: 13-SEP-1990

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 918 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola lanuginosa

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..873

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG AGG AGC TCC CTT GTG CTG TTC TTT GTC TCT GCG TGG ACG GCC TTG   48
Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
 1               5                  10                  15

GCC AGT CCT ATT CGT CGA GAG GTC TCG CAG GAT CTG TTT AAC CAG TTC   96
Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
            20                  25                  30

AAT CTC TTT GCA CAG TAT TCT GCA GCC GCA TAC TGC GGA AAA AAC AAT  144
Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
        35                  40                  45

GAT GCC CCA GCT GGT ACA AAC ATT ACG TGC ACG GGA AAT GCC TGC CCC  192
Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
    50                  55                  60

GAG GTA GAG AAG GCG GAT GCA ACG TTT CTC TAC TCG TTT GAA GAC TCT  240
Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
65                  70                  75                  80

GGA GTG GGC GAT GTC ACC GGC TTC CTT GCT CTC GAC AAC ACG AAC AAA  288
Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
                85                  90                  95

TTG ATC GTC CTC TCT TTC CGT GGC TCT CGT TCC ATA GAG AAC TGG ATC  336
Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
            100                 105                 110

GGG AAT CTT AAC TTC GAC TTG AAA GAA ATA AAT GAC ATT TGC TCC GGC  384
Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
        115                 120                 125

TGC AGG GGA CAT GAC GGC TTC ACT TCG TCC TGG AGG TCT GTA GCC GAT  432
Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
    130                 135                 140
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ACG TTA AGG CAG AAG GTG GAG GAT GCT GTG AGG GAG CAT CCC GAC TAT  480
Thr L u Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
145                 150                 155                 160

CGC GTG GTG TTT ACC GGA CAT AGC TTG GGT GGT GCA TTG GCA ACT GTT  528
Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
                165                 170                 175

GCC GGA GCA GAC CTG CGT GGA AAT GGG TAT GAT ATC GAC GTG TTT TCA  576
Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
            180                 185                 190

TAT GGC GCC CCC CGA GTC GGA AAC AGG GCT TTT GCA GAA TTC CTG ACC  624
Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
        195                 200                 205

GTA CAG ACC GGC GGA ACA CTC TAC CGC ATT ACC CAC ACC AAT GAT ATT  672
Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
    210                 215                 220

GTC CCT AGA CTC CCG CCG CGC GAA TTC GGT TAC AGC CAT TCT AGC CCA  720
Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
225                 230                 235                 240

GAG TAC TGG ATC AAA TCT GGA ACC CTT GTC CCC GTC ACC CGA AAC GAT  768
Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
                245                 250                 255

ATC GTG AAG ATA GAA GGC ATC GAT GCC ACC GGC GGC AAT AAC CAG CCT  816
Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
            260                 265                 270

AAC ATT CCG GAT ATC CCT GCG CAC CTA TGG TAC TTC GGG TTA ATT GGG  864
Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
        275                 280                 285

ACA TGT CTT TAGTGGCCGG CGCGGCTGGG TCCGACTCTA GCGAGCTCGA GATCT   918
Thr Cys Leu
    290
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 291 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
 1               5                  10                  15

Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
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                20                  25                  30
Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
            35                  40                  45
Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
        50                  55                  60
Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
65                  70                  75                  80
Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
                85                  90                  95
Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
            100                 105                 110
Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
        115                 120                 125
Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
    130                 135                 140
Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
145                 150                 155                 160
Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
                165                 170                 175
Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
            180                 185                 190
Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
        195                 200                 205
Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
    210                 215                 220
Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
225                 230                 235                 240
Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
                245                 250                 255
Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
            260                 265                 270
Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
        275                 280                 285
Thr Cys Leu
    290
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CLAIMS

1. A lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, wherein the electrostatic charge and/or hydrophobicity of a lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues may participate in the interaction with the substrate at or during hydrolysis, has been changed by deleting or substituting one or more negatively charged amino acid residues by neutral or positively charged amino acid residue(s), and/or by substituting one or more neutral amino acid residues by positively charged amino acid residue(s), and/or by deleting or substituting one or more hydrophilic amino acid residues by hydrophobic amino acid residue(s).

2. A lipase variant according to claim 1, wherein one or more glutamic acid aspartic acid residues of said lipid contact zone are substituted by glutamine, asparagine, alanine, leucine, valine, serine, threonine, lysine, or arginine.

3. A lipase variant according to any of claims 1-2, wherein the parent lipase is a microbial lipase.

4. A lipase variant according to claim 3, wherein the parent lipase is a fungal lipase.

5. A lipase variant according to claim 4, wherein the parent lipase is derived from a strain of Humicola, or Rhizomucor.

6. A lipase variant according to claim 5, wherein the parent lipase is a Rhizomucor miehei lipase.

7. A lipase variant according to claim 6, wherein one or more amino acid residues are substituted as follows:
D91N,K,R,A,V,L,S,T;
D256N,K,R,A,V,L,S,T;
D226N,K,R,A,V,L,S,T;
D61N,K,R,A,V,L,S,T;
D113N,K,R,A,V,L,S,T;
E201Q,K,R,A,V,L,S,T;
D243N,K,R,A,V,L,S,T.

8. A lipase variant according to claim 5, wherein the parent lipase is a Humicola lanuginosa lipase.

9. A lipase variant according to claim 8, wherein one or more amino acid residues are substituted as follows:
E87Q,K,R,A,N,T,S,L,V;
D254N,K,R,A,Q,T,S,L,V;
D242N,K,R,A,Q,T,S,L,V;
E210Q,K,R,A,N,T,S,L,V;
E56Q,K,R,A,N,T,S,L,V;
D96N,K,R,A,Q,T,S,L,V;
D111N,K,R,A,Q,T,S,L,V;
D62A,Q,N,T,S,K,R,L,V;
E219A,Q,N,T,S,K,R,L,V;
E234A,Q,N,T,S,K,R,L,V;
E57A,Q,N,T,S,K,R,L,V
E99A,Q,N,T,S,K,R,L,V;
D27A,Q,N,T,S,K,R,L,V; or
E239A,Q,N,T,S,K,R,L,V.

10. A lipase variant according to claim 9, wherein one or more amino acid residues are substituted as follows:
E87Q + D254N + D242N + E210Q;
E87Q + D254N + E210Q;
D96N + E87Q + D254N;
R209A + E210A.

11. A lipase variant according to claim 8, wherein one or more amino acid residues are substituted as follows
T267K,R;
S85K,R;
T226K,R;
N88K,R;
N92K,R;
I255K,R;
I202K,R
L206K,R;
L259K,R;
V203K,R; or
L227K,R

12. A lipase variant according to claim 4, wherein the parent lipase is a yeast lipase.

13. A lipase variant according to claim 12, wherein the parent lipase is derived from a strain of Candida.

14. A lipase variant according to claim 3, wherein the parent lipase is a bacterial lipase.

15. A lipase variant according to claim 14, wherein the parent lipase is derived from a strain of Pseudomonas.

16. A lipase variant comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, said lipase variant being characterized by substitution, deletion, or insertion of one or more amino acid residues at the position of one or more of the amino acid residues constituting the sequence of the lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues participate in the interaction with the substrate at or during hydrolysis so as to change the surface conformation of said lipid contact zone.

17. A lipase variant according to claim 16, wherein one or more amino acid residues are substituted by one or more other, less bulky amino acid residues.

18. A lipase variant according to claim 17, wherein one or more amino acid residues are substituted by valine, threonine, serine, glycine, or alanine.

19. A lipase variant according to claim 16, wherein one or more amino acid residues forming part of loop sequences in the lipid contact zone are deleted.

20. A lipase variant according to claim 19, wherein 2-8, in particular 2-6 amino acid residues are deleted from one or more loop sequences in the lipid contact zone.

21. A lipase variant according to any of claims 16-20, wherein the parent lipase is a microbial lipase.

22. A lipase variant according to claim 21, wherein the parent lipase is a fungal lipase.

23. A lipase variant according to claim 22, wherein the parent lipase is derived from a strain of Humicola or Rhizomucor.

24. A lipase variant according to claim 23, wherein the parent lipase is a Rhizomucor miehei lipase.

25. A lipase variant according to claim 24, wherein one or more amino acid residues are substituted as follows:
I204V,A,T,S,G;
L208V,A,T,S,G;
F213V,A,T,S,G; or
I254V,A,T,S,G

26. A lipase variant according to claim 24, wherein one or more amino acid residues are deleted at one or more of the following positions: 82-113, 211-215, 235-243, 245-269 or 264-269.

27. A lipase variant according to claim 26, which is modified as follows:
N264* + T265* + G266* + L267* + C268* + T269* + C22T.

28. A lipase variant according to claim 26, wherein the following amino acid residues are deleted:
F213* + F215*;
D238* + L239* + E240* + D243*; or
F251* + T252* + S247*

29. A lipase variant according to claim 23, wherein the parent lipase is a Humicola lanuginosa lipase.

30. A lipase variant according to claim 29, wherein one or more amino acid residues are substituted as follows:
I202V,A,T,S,G;
L206V,A,T,S,G;
F211V,A,T,S,G,I; or
I255V,A,T,S,G

31. A lipase variant according to claim 29, wherein the one or more amino acid residues are deleted at one or more of the following positions: 84-112, 209-213, 238-245, 247-254 or 264-269.

32. A lipase variant according to claim 31, which is modified as follows:
L264* + I265* + G266* + T267* + C268* + L269* + C22T.

33. A lipase variant according to claim 31, wherein the following amino acid residues are deleted:
R209* + E210*;
F211* + Y213*;

D242* + E239* + I241*; or
N247* + D254*

34. A lipase variant according to claim 21, wherein the parent lipase is a yeast lipase.

35. A lipase variant according to claim 34, wherein the parent lipase is a derived from a strain of Candida.

36. A lipase variant according to claim 21, wherein the parent lipase is a bacterial lipase.

37. A lipase variant according to claim 36, wherein the parent lipase is derived from a strain of Pseudomonas.

38. A lipase variant of a type comprising (i) a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule and (ii) a surface loop structure which covers the active serine when the lipase is in inactive form and which changes its conformation when the lipase is activated so as to make the active serine accessible to a lipid substrate, the loop structure having a predominantly hydrophobic inner surface facing the binding pocket and a predominantly hydrophilic outer surface, said lipase variant being characterized by substitution, deletion or insertion of one or more amino acid residues at the position of one or more of the amino acid residues constituting the sequence of the loop structure and/or constituting the sequence of the lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues participate in either shifting the surface loop structure or the interaction which the substrate at or during hydrolysis.

39. A lipase variant according to claim 38, wherein at least one amino acid residue of the loop structure is substituted by cysteine, and wherein at least one other amino acid residue is

substituted by cysteine, the two cysteine residues being so positioned relative to each other as to form a disulphide bond.

40. A lipase variant according to claim 38 or 39, wherein the parent lipase is a microbial lipase.

41. A lipase variant according to any of claims 38-40, wherein the parent lipase is a fungal lipase.

42. A lipase variant according to claim 41, wherein the parent lipase is derived from a strain of Humicola or Rhizomucor.

43. A lipase variant according to claim 42, wherein the parent lipase is a Rhizomucor miehei lipase.

44. A lipase variant according to claim 43, wherein one or more amino acid residues are substituted as follows:
S114C + A90C;
R86C + D61C;
S84C + D61C;
N87C + D61C;
Y60C + R78C; or
Y60C + N87C

45. A lipase variant according to claim 43, wherein one or more amino acid residues are substituted as follows:
I204V,T,S,A,G;
L208V,T,S,A,G;
V254V,T,S,A,G;
L255V,T,S,A,G;
L258V,T,S,A,G;
L267V,T,S,A,G;
F94L,T,K; or
F213L,T,K

46. A lipase variant according to claim 43, which comprises the following amino acid substitutions:

I204T + L255T + L267T; or
L208T + V254T + L258T

47. A lipase variant according to claim 42, wherein the parent lipase is a Humicola lanuginosa lipase.

48. A lipase variant according to claim 47, wherein one or more amino acid residues are substituted as follows:
G61C + N88C;
G61C + E87C;
D62C + E87C;
D62C + S85C;
D62C + N88C; or
S116C + G91C

49. A lipase variant according to claim 47, wherein one or more amino acid residues are substituted as follows:
L93V,T,S,A,G;
I90V,T,S,A,G;
I86V,T,S,A,G;
I202V,T,S,A,G;
L206V,T,S,A,G;
I255V,T,S,A,G;
L259V,T,S,A,G;
I265V,T,S,A,G;
F95L,T,K; or
F211L,T,K

50. A lipase variant according to claim 49, wherein one or more amino acid residues are substituted as follows:
F95K;
I86T;
I90T;
I255T;
L259T;
L206T; or
L206T + I255T + L259T

51. A lipase variant according to claim 40, wherein the parent lipase is a yeast lipase.

52. A lipase variant according to claim 51, wherein the parent lipase is derived from a strain of Candida.

53. A lipase variant according to claim 40, wherein the parent lipase is a bacterial lipase.

54. A lipase variant according to claim 53, wherein the parent lipase is derived from a strain of Pseudomonas.

55. A DNA construct comprising a DNA sequence encoding a lipase variant according to any of claims 1-54.

56. A recombinant expression vector which carries a DNA construct according to claim 55.

57. A cell which is transformed with a DNA construct according to claim 55 or a vector according to claim 56.

58. A cell according to claim 57 which is a fungal cell, e.g. belonging to the genus Aspergillus, such as A. niger, A. oryzae, or A. nidulans; a yeast cell, e.g. belonging to a strain of Saccharomyces, such as S. cerevisiae, or a methylotrophic yeast from the genera Hansenula, such as H. polymorpha, or Phichia, such as P. pastoris; or a bacterial cell, e.g. belonging to a strain of Bacillus, such as B. subtilis, or B. lentus.

59. A cell according to claim 57 which is a plant cell, e.g. belonging to the Solanaceae, such as Solanum tuberosum, or Nicotiana tabacum.

60. A plant comprising a cell according to claim 59.

61. A method of producing a lipase variant according to any of claims 1-54, wherein a cell according to any of claims 57-59, or

a plant according to claim 60 is cultured or grown under conditions conducive to the production of the lipase variant, and the lipase variant is subsequently recovered from the culture or plant.

62. A detergent additive comprising a lipase variant according to any of claims 1-54, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme.

63. A detergent additive according to claim 62 which contains 0.02-200 mg of enzyme protein/g of the additive.

64. A detergent additive according to claim 62 or 63 which additionally comprises another enzyme such as a protease, amylase, peroxidase and/or cellulase.

65. A detergent composition comprising a lipase variant according to any of claims 1-54.

66. A detergent composition according to claim 65 which additionally comprises another enzyme such as a protease, amylase, peroxidase and/or cellulase.

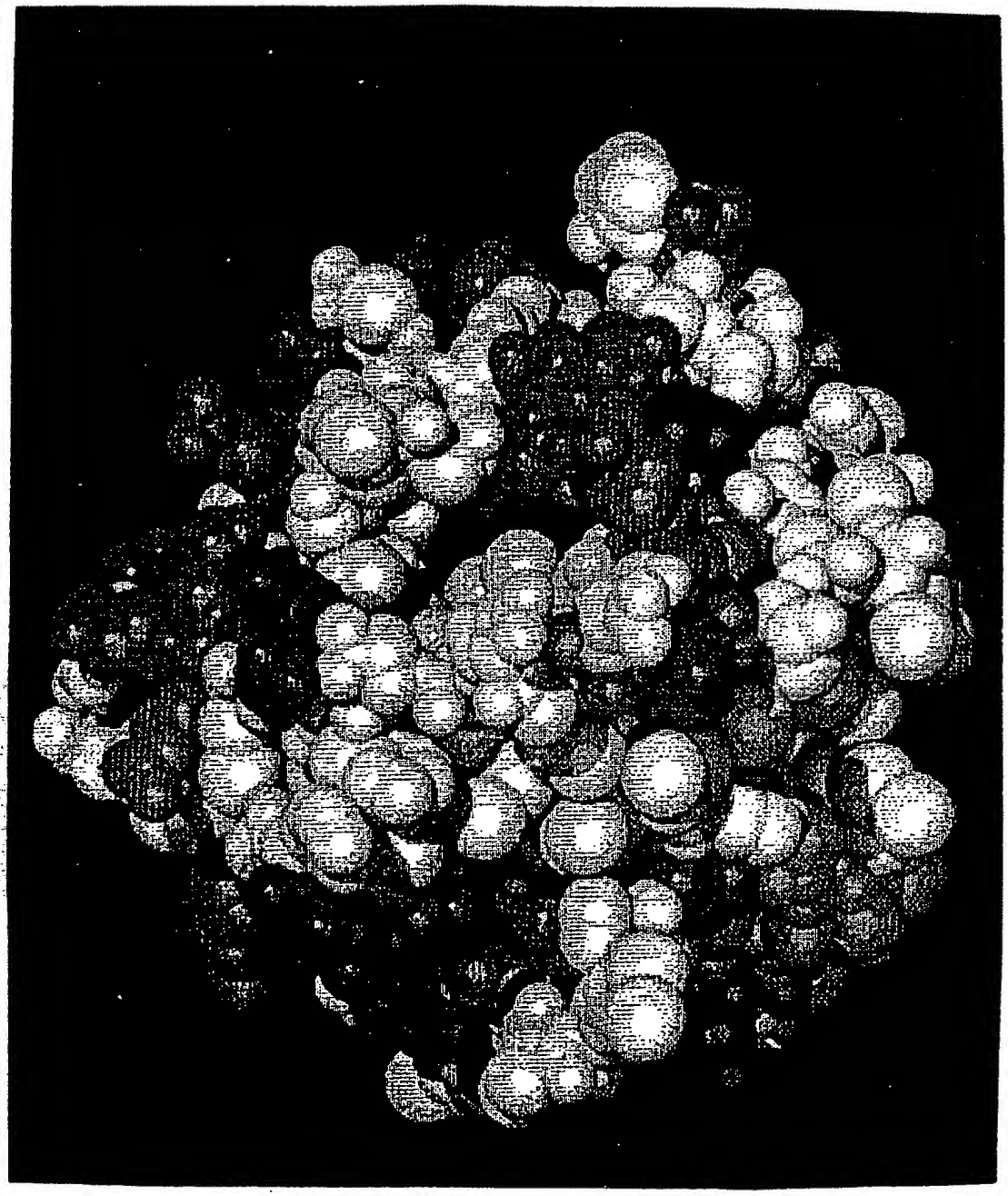


Fig. 1a

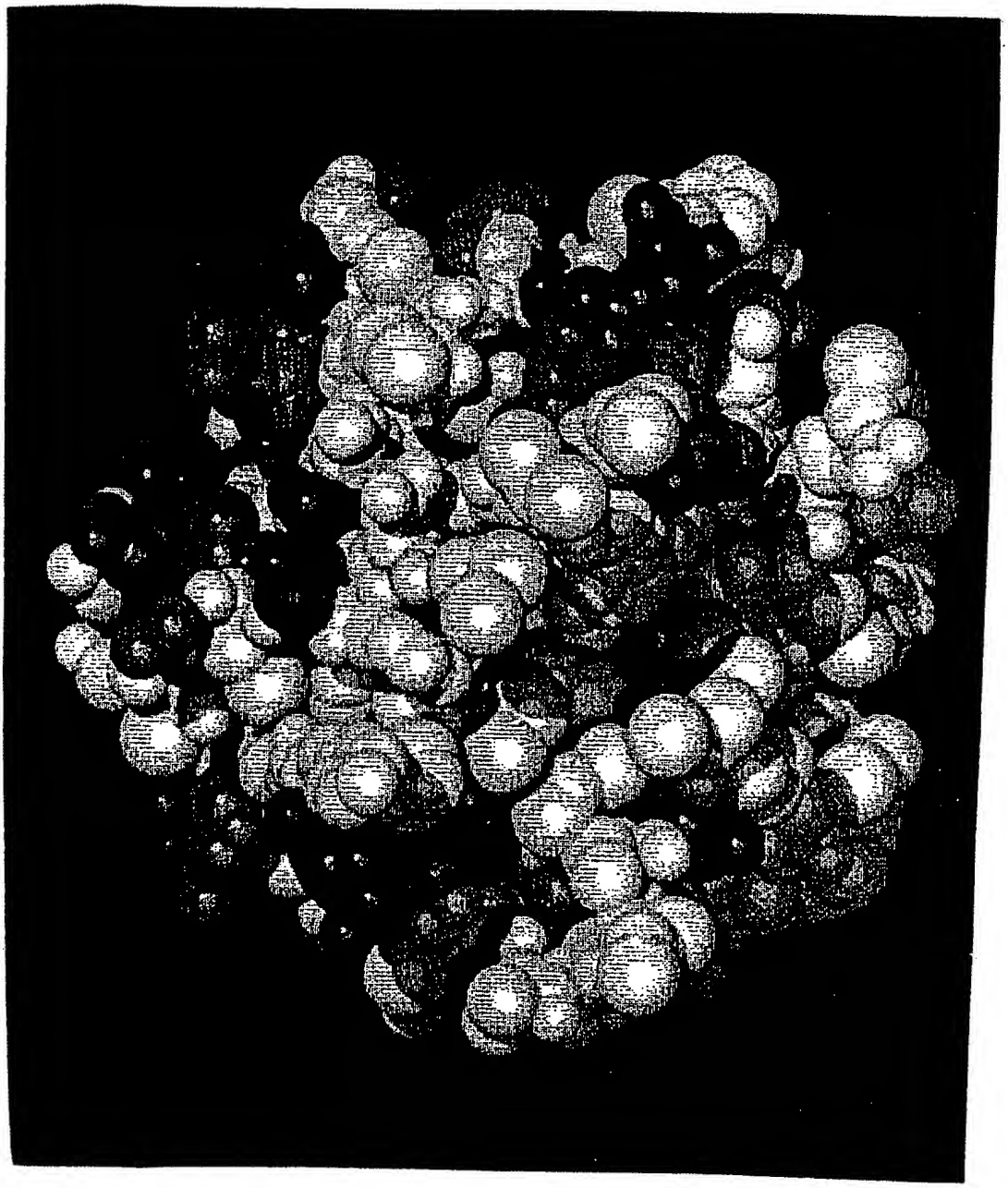


Fig. 1b

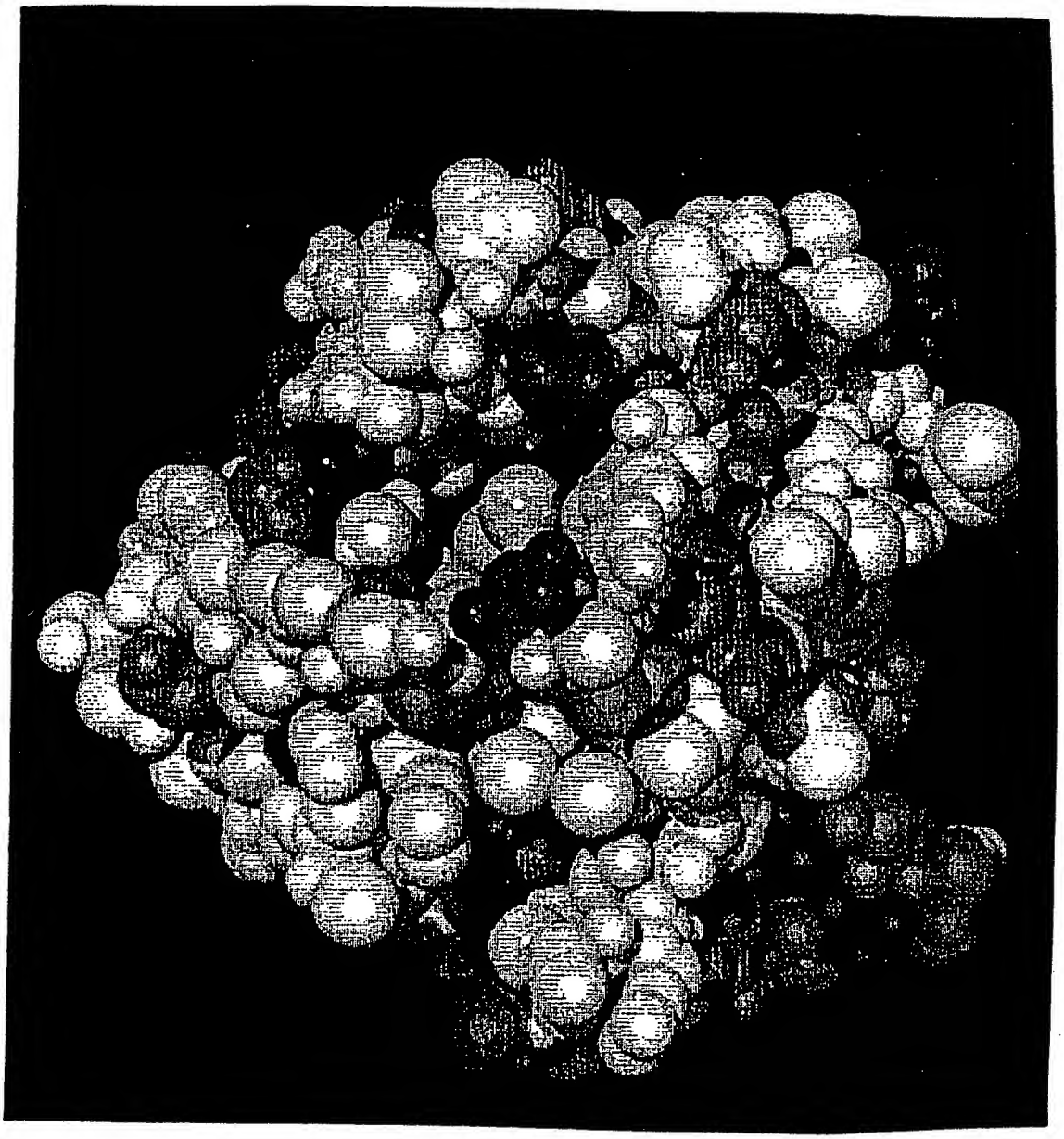


Fig. 2a

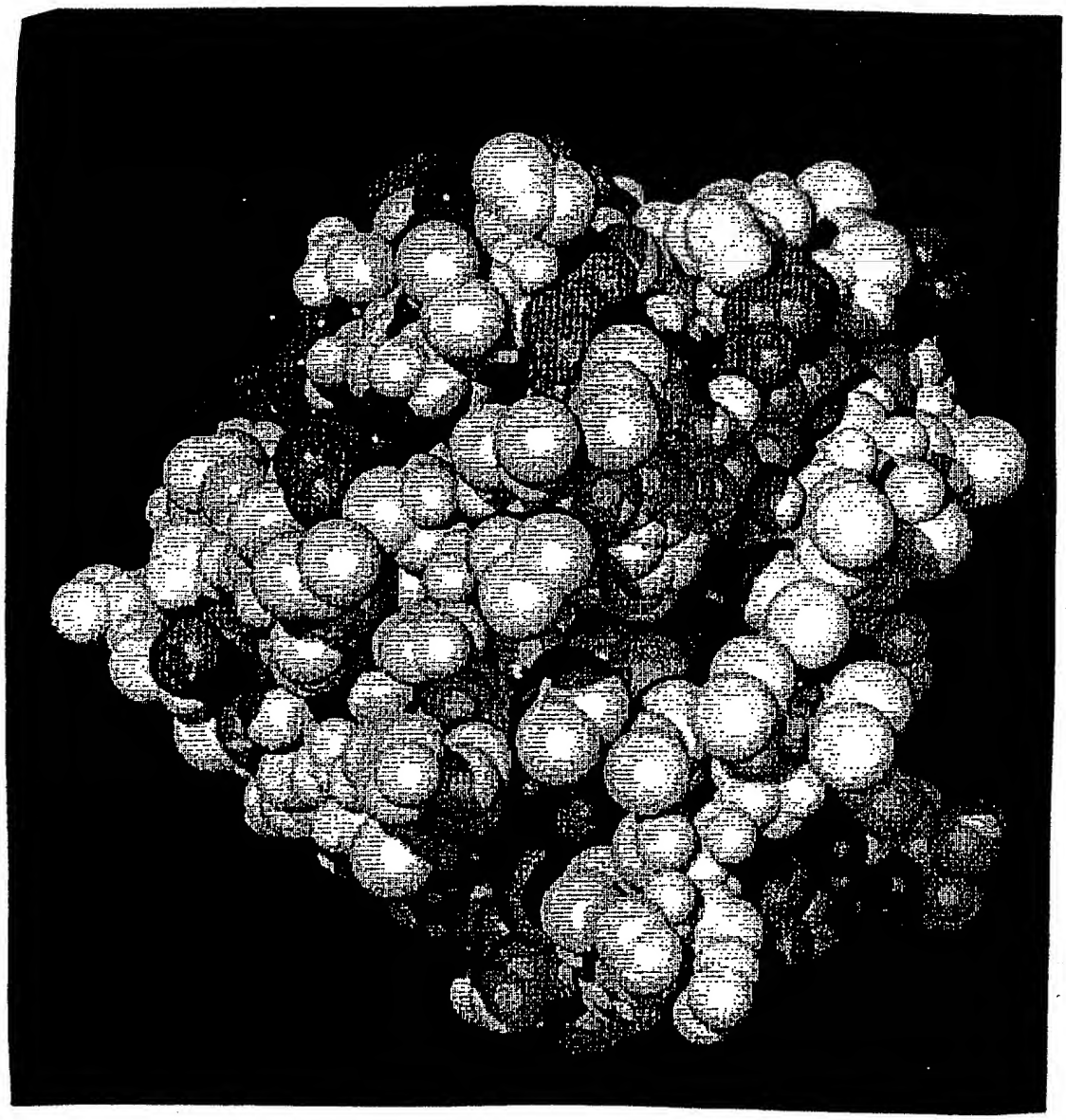


Fig. 2b

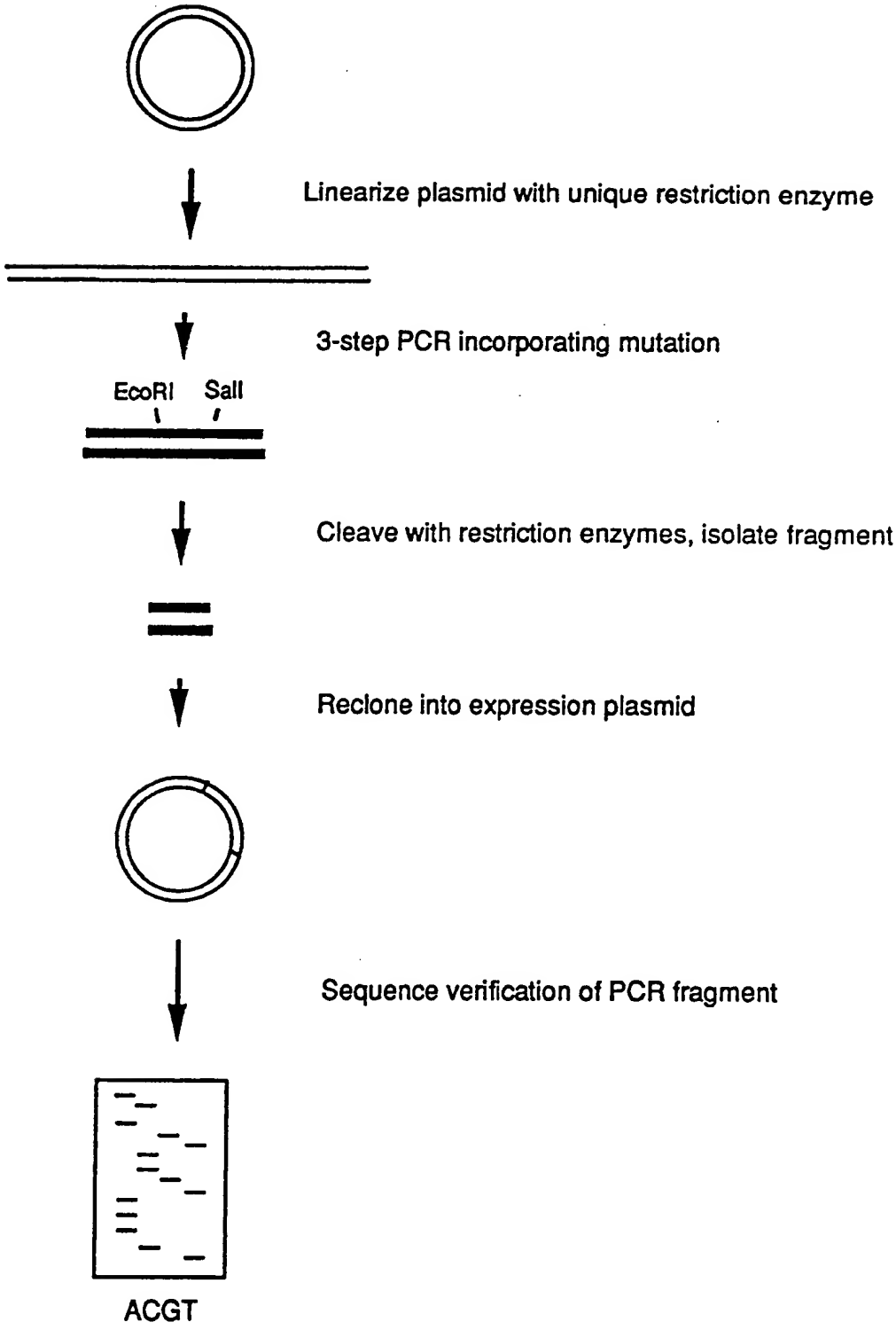

Linearize plasmid with unique restriction enzyme
3-step PCR incorporating mutation
EcoRI
SalI
Cleave with restriction enzymes, isolate fragment
Reclone into expression plasmid
Sequence verification of PCR fragment
ACGT

Fig. 3

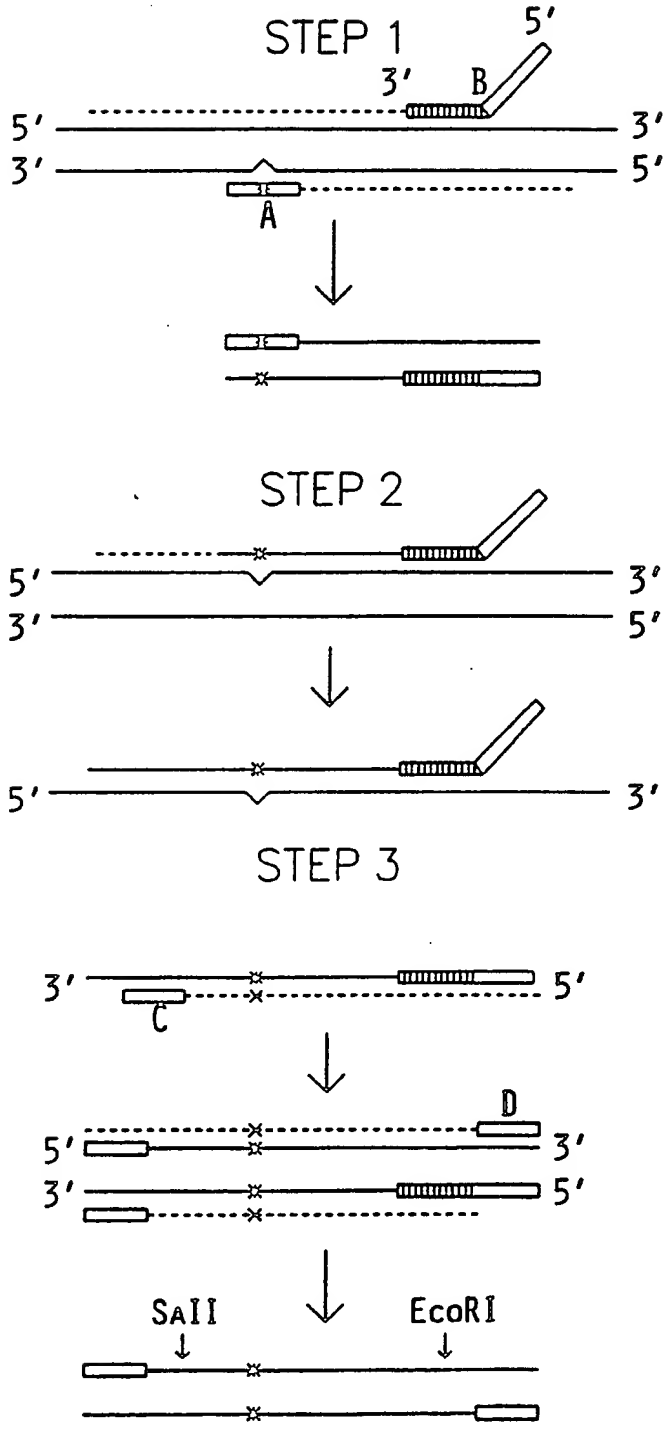
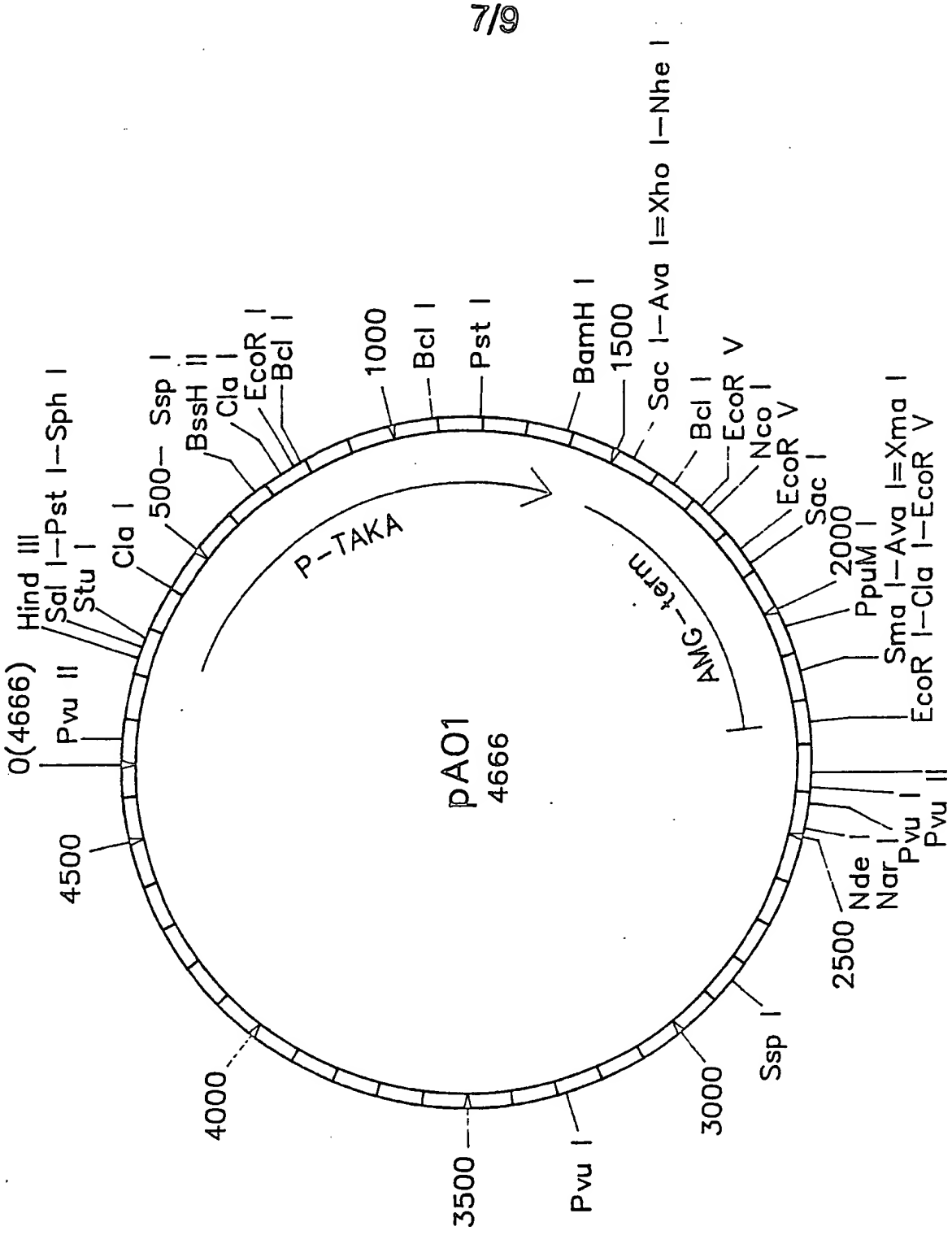


Fig. 4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Hind III
Sal I—Pst I—Sph I
Stu I
Cla I
500—
Ssp I
BssH II
Cla I
EcoR I
Bcl I
1000
Bcl I
Pst I
BamH I
1500
Sac I—Ava I=Xho I—Nhe I
Bcl I
EcoR V
Nco I
EcoR V
Sac I
2000
PpuM I
Sma I—Ava I=Xma I
EcoR I—Cla I—EcoR V
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Pvu I
Nar I
Nde I
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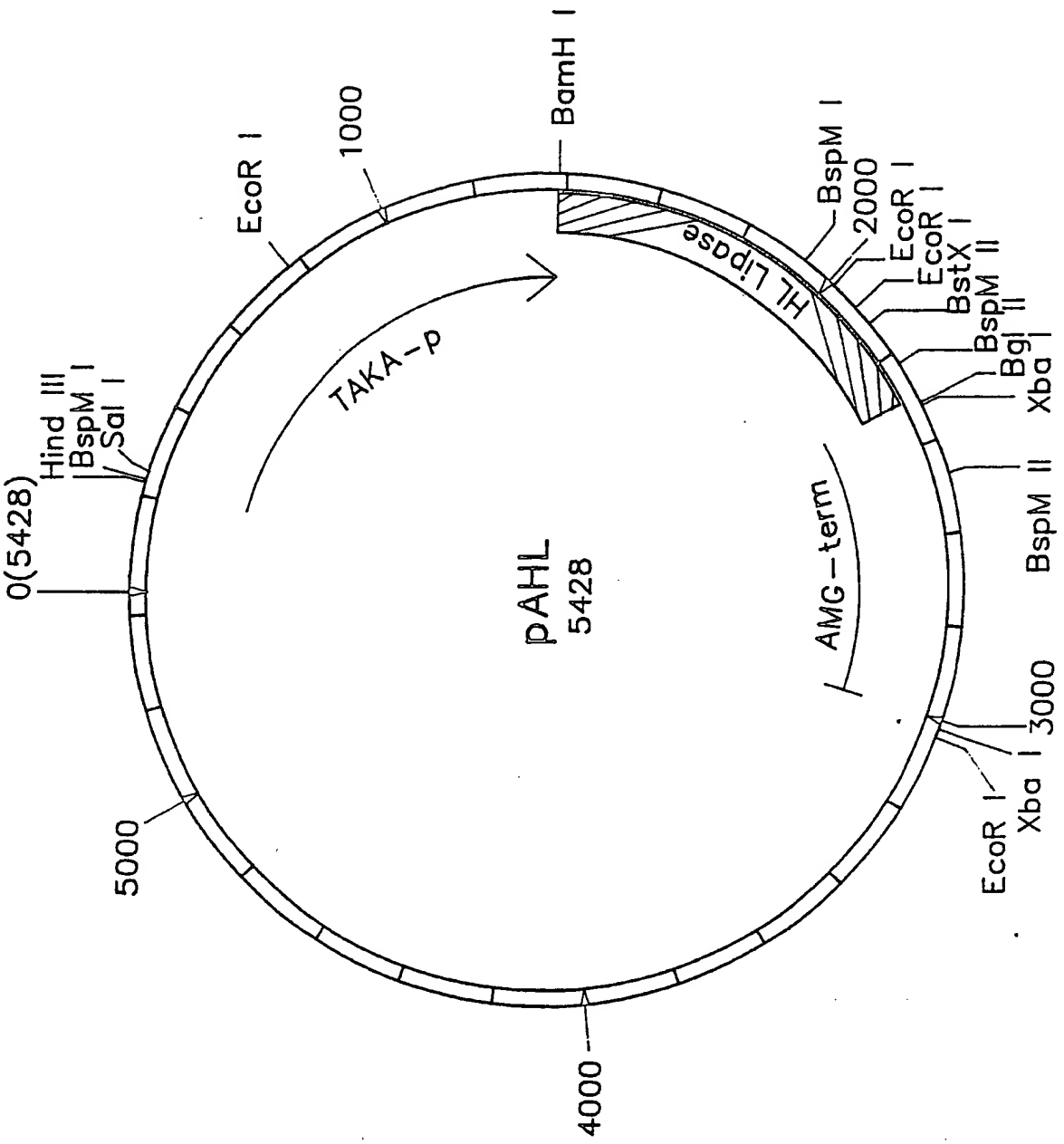
Ssp I
3000
Pvu I
3500
4000

Fig. 5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Xba I
Bgl II
BspM II
BstX I
EcoR I
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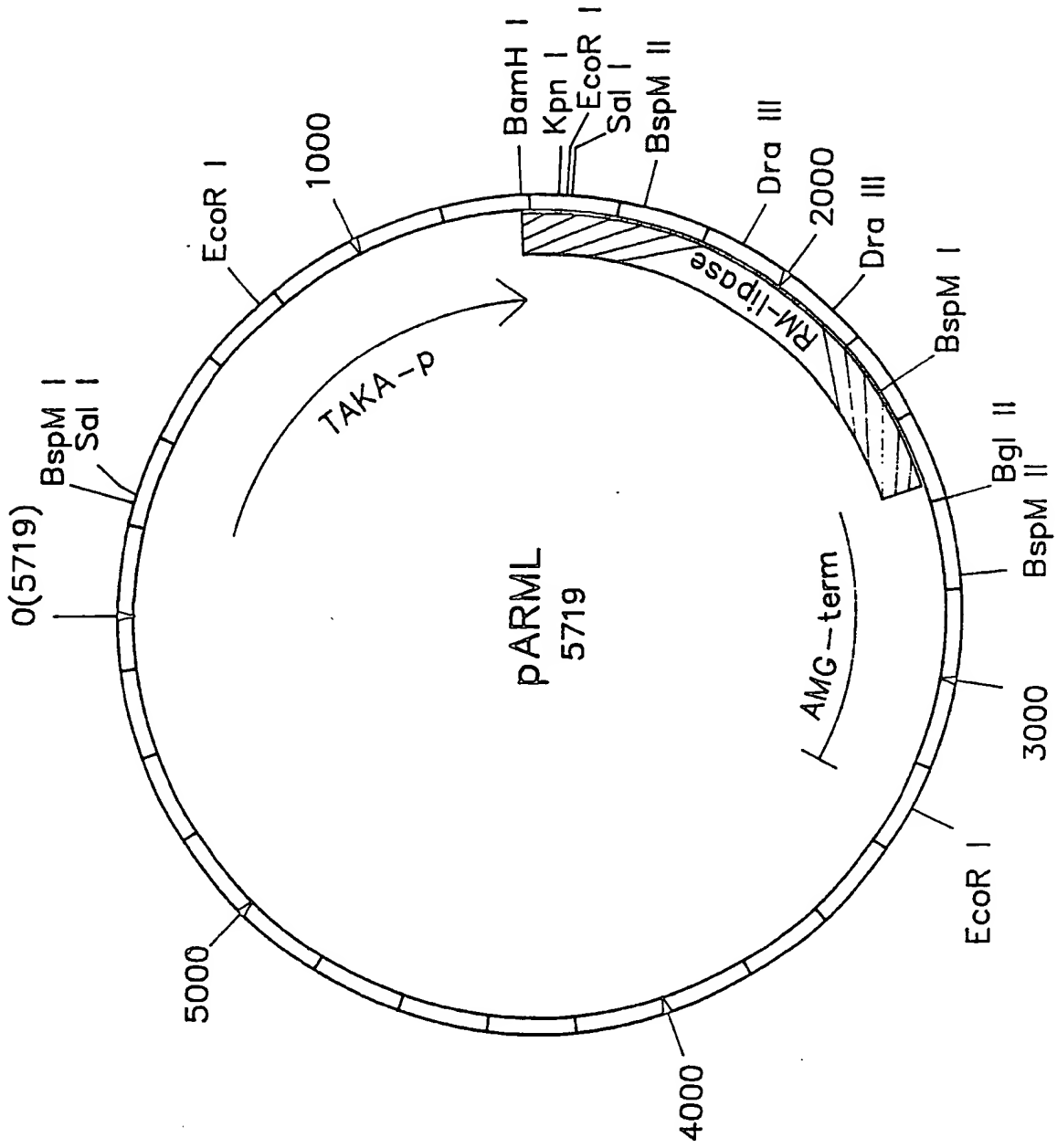
HL Lipase
TAKA-P
AMG-term
pAHL
5428

Fig. 6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AMG-term
BamH I
Kpn I
EcoR I
Sal I
BspM II
Dra III
Dra III
BspM I
Bgl II
BspM II
EcoR I
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4000
5000
0(5719)
BspM I
Sal I
EcoR I
1000
2000

Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00271

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 9/20, 15/55, C 11 D 3/386, C 12 N 5/14

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; C 11 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	EP, A1, 0407225 (UNILEVER PLC) 9 January 1991, see abstract --	1,3,14-16,21,36-37,55-66
X	EP, A2, 0375102 (THE CLOROX COMPANY) 27 June 1990, see especially claim 11 Table II --	1,3,14-17,21,36-37,55-66
A	EP, A1, 0305216 (NOVO INDUSTRI A/S) 1 March 1989, see the whole document --	1-66
X	Dialog Information Services, File 154, Medline 85-92, Dialog accession no. 07295905, Davis RC et al: "Hepatic lipase: site-directed mutagenesis of a serine residue important for catalytic activity", J Biol Chem, Apr 15 1990, 265 (11) p 6291-5 --	1,3,16,55-66

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13th February 1992	1992 -02- 14
International Searching Authority: SWEDISH PATENT OFFICE	Signature of Authorized Officer: Mikael G:son Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Dialog Information Services, File 154, Medline 85-92, Dialog accession no. 07295773, Semenkovich CF et al: "In vitro expression and site-specific mutagenesis of the cloned human lipoprotein lipase gene. Potential N-linked glycosylationsite asparagine 43 is important for both enzyme activity and secretion" J Biol Chem Apr 5 1990, 265 (10) p 5429-33 --	1,3,16, 55-66
X	Dialog Information Services, File 357, Biotechnology abstracts, Dialog accession no. 077832, Poulose A J: "Alteration of substrate specificity of a lipase by site specific mutagenesis - Pseudomonas enzyme (conference abstract)", Abstr.Pap.Am.Chem.Soc. (195 Meet BTEC47) 1988 --	1,3,16, 55-66
A	NATURE, Vol. 343, 1990 Leo Brady et al: "A serine protease triad forms the catalytic centre of a triacylglycerol lipase", see page 767 - page 770 --	1-66
A	NATURE, Vol. 343, 1990 F.K. Winkler et al: "Structure of human pancreatic lipase", see page 771 - page 774 --	1-66

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet!

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional seach fees.

Claims 1-15 completely, 55-66 partially.

A lipase variant, where the electrostatic charge and/or the hydrophobicity of the lipid contact zone has been changed.

claims 16-37 completely, 55-66 partially.

A lipase variant, where the surface conformation of the lipid contactzone has been changed

claims 38-54 completely 55-66 partially.

A lipase variant supplied with a surface loop structure, which covers the active serine when the lipase is inactive, and which changes its conformation when the lipase is activated so as to make the active serine accessible to a lipid substrate.

These three categories of lipase variants are not considered have enough technical relationship so as to form a single inventive concept, particularly since it is known by EP,A2, 375 102 to change certain aminoacid residues of a lipase from Pseudomonas putida.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00271

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 30/11/91 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0407225	91-01-09	WO-A- 91/00910	91-01-24
EP-A2- 0375102	90-06-27	JP-A- 2225599	90-09-07
EP-A1- 0305216	89-03-01	JP-A- 1157383	89-06-20